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Expression and Production of Human Granulocyte Colony Stimulating Factor (G-CSF) in Silkworm Cell Line

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Granulocyte colony stimulating factor (G-CSF) is a hematopoietic cytokine that stimulates bone marrow cells to proliferate and differentiate into granulocytes. G-CSF is approved and used for therapeutic purposes. The endoplasmic reticulum (ER) signal peptide of hG-CSF was replaced with silkworm-specific signal peptides to express and efficiently secrete recombinant hG-CSF by silkworm cells. Plasmids that contain cDNAs for hG-CSF and hG-CSF fused with silkworm- specific signal peptides of prophenoloxidase activating enzyme (PPAE), protein disulfide isomerase (PDI), and bombyxin (BX) were constructed. The G-CSF protein was expressed in insect cell line BM5 and was detected by western blot analysis. The cells transfected with plasmids containing rhG-CSF genes with silkworm-specific signal sequences released mature rhG-CSF protein more efficiently than the cells transfected with pG-CSF, the plasmid containing human G-CSF gene, including its own signal sequence. The production of hG-CSF reached maximal level at four days post-transfection and remained at a high level until 7 days post-transfection. These data demonstrate that the modification of the human G-CSF mimic to insect proteins synthesized in ER greatly improves the production of the protein.

Key words : G-CSF, insect cells, ER signal sequence, recombinant protein

Introduction

Colony stimulating factors (CSFs) stimulate bone marrow cells in culture to generate colonies of hematopoietic cells [9]. Among CSFs, a glycoprotein that can generate granulocytic colonies specifically is granulocyte colony stimulating factor (G-CSF). G-CSF stimulates the bone marrow cells to proliferate and differentiate into granulocytes and hematopoietic stem cells. G-CSF also stimulates proliferation, differentiation and activation of the precursor of neutrophil cells [8]. Cancer patients undergo chemotherapy often encountered decrease in white blood cell count and this affects the patient's immune system. The recombinant G-CSF produced in *E. coli* was able to generate macrophages and granulocytes from human leukemia cells [12]. G-CSF was approved for several therapeutic applications including treatment of neutropnenia and acute myeloid leukemias [6,11,14,16].

The precursor protein of G-CSF has 204 amino acids that include 30 amino acids of ER-signal sequence. The mature protein consists of 174 amino acids and contains O-linked glycosylation at threonine 133 [4]. Recombinant human G-CSF (rhG-CSF) has been produced in genetically engineered *Escherichia coli* (Filgrastim) and has been approved for use in cancer patients who need high-dose chemotherapy [3,15]. rhG-CSF was also produced in eukaryotic cells to generate a glycosylated form. Lenograstim is a glycosylated form of G-CSF produced in Chinese Hamster Ovary cells (CHO) [7]. The glycosylations of G-CSF contribute to its stability [2,10]. Lenograstim is more active than Filgrastim, a non-glycosylated G-CSF [5]. Because the post-translational modification of a precursor protein in prokaryotic cells is different from eukaryotic cells, eukaryotic cells have been used to produce recombinant human therapeutic drugs. The secreted rhG-CSF in insect cells is more advantageous than the protein produced in *E.coli* in purification process since the protein in *E. coli* is located in inclusion bodies [13,17].

We cloned cDNA for hG-CSF in an expression vector for the production of proteins in insect cells. We also subcloned cDNA for hG-CSF in which the signal peptide was replaced with that from silkworm enzymes, which are secreted proteins of silkworm. The expression and production of the rhG-CSF were described.

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Materials and Methods

Cell culture and transfection

BM5 cells were cultured in GRACE medium (Welgene) containing 5% fetal calf serum (Gibco) containing 1× penicillin, streptomycin, and amphotericin B (Gibco Anti-Anti, Invitrogen Corporation) under 5% CO_2. BM5 cells were plated at 4×10^5 cells per 25 cm^2 tissue culture plate. The transfection was performed using the FuGENE® HD Transfection Reagent (Roche) according to the procedure provided by the manufacturer. The medium was replaced with new medium after 24 hr incubation.

Construction of G-CSF expression plasmid

G-CSF cDNA clone (ATCC 10468871) was purchased from American type culture collection (ATCC). Human G-CSF coding sequence was amplified using the G-CSF cDNA clone as a template, the forward primer JA6, and the reverse primer JB6 including 3′end of cDNA of hG-CSF with deletion of a termination codon (Table 1). The PCR amplified DNA fragment was digested with Sac and AgeI and subcloned into pIZTv5/his vector.

The plasmids containing human G-CSF coding sequence with silkworm specific ER-signal sequences were constructed. The fusion cDNA of hG-CSF and silkworm specific ER-signal sequences from prophenoloxidase activating enzyme (PPAE), protein disulfide isomerase (PDI), and bombyxin (BX) were amplified by two-step PCR. Mature human G-CSF coding sequence was amplified first using pGCSF as a template, the primer JA6F which include the 5′ end of cDNA coding for mature hG-CSF, and the reverse primer JB6 including 3′end of cDNA of hG-CSF. The cDNA of hG-CSF with PPAE signal sequence was then amplified with the first PCR reaction as a template, the primer JA4F including the coding sequence for the signal sequence of PPAE, and the primer JB6. The coding sequence for hG-CSF with ER-signal sequence of PDI were amplified similarly using the primer set of JA10 and JB6 for the first PCR and the primers JA9 and JB6 for the second PCR. The coding sequence for hG-CSF with ER-signal sequence of BX were amplified using the primers JA12 and JB6 for the first PCR and primers JA11 and JB6 for the second PCR. The PCR-amplified DNA fragments are restricted by XbaI and BglII and ligated with pIZTv5/his vector cut with the same enzymes.

Signal peptide prediction program SignalP 3.0 Server [1] (http://www.cbs.dtu.dk/services/SignalP/) were used to predict and ER signal sequence for fusion hG-CSF and selected the sequence showed proper signal peptide cleavage.

Batch purification of rhG-CSF

Four days after transfection of rhG-CSF expression plasmids the culture medium was harvested. After centrifuge for 5 min at 1,500 rpm in a bench top centrifuge. The supernatant was collected. Two ml of the supernatant was adjusted to a condition of 1x binding buffer using 10× binding buffer (100 mM sodium phosphate buffer, 500 mM NaCl, 100 mM imidazole, pH 7.4). Sixty μl of 50% slurry of nickel sepharose beads (Pharmacia) were added to 2 ml of culture

Table 1. Primer sequences

Name	Nucleotide sequence	Region
JA6	ATA CTG AGC TCT CTA GAA TGT CTC CTG AGC CCG CTC TG	5′ of G-CSF precursor
JA4-F	CCG AGC TCT CTA GAA TGT TTT TAA TTT GGA CAT TCA TCG TGG CTG TTC TGG CGA TCC AGA CCA AAA GT	5′ of PPAE G-CSF
JA6-F	CTG GCG ATC CAG ACC AAA AGT GTT GTT GCA ACC CCC CTG GGC CCT GCC	5′ of PPAE G-CSF
JB6	ATC TGA CCG GTA GAT CTC CCG ACC CGT TCC ACC GCG GGC TGG GCA AGG TGG CG	3′ of G-CSF
JA9	TAT TAG AAT TCG GAT CCG AAA TGT TCG GAT CAC TAA AGT TTG TTC TTT TAT TGG GCA TA	5′ of PDI
JA10	ATT GGG CAT AAT TTA TTT ATG TAA AGC GAC CCC CCT GGG CCC TGC C	5′ of PDI and G-CSF
JA11	TAT TAG AAT TCG GAT CCG AAA TGA TGA AGA CTG CAG TAA TGT TCA TAT TAG TAG TCG TGA TCA G	5′ od BX
JA12	GTC GTG ATC AGT TTG ACG TAT TCA ACC CCC CTG GGC CCT GCC	5′ of BX and G-CSF
JB8	ATA TAC CGG TCT CGA GGG GCT GGG CAA GGT GGC G	3′ of GCSF

supernatant, rotate for an hour at 4°C, washed for 5 min for 3 times. The final nickel beads were suspended with 30 μl of 2× gel loading buffer and used for PAGE gel analysis.

Polyacrylamide gel electrophoresis and Western blot analysis of rhG-CSF

Samples were boiled for 5 min and 20 μl of each samples were applied to each lane of a 12%-15% polyacrylamide gel. The gels electrophoresis was run at 100 volts for an hour and the gels were blotted to PROTRAN nitrocellulose membrane (Whatman). Membranes were blocked with gelatin wash buffer (0.1% gelatin, 15mM Tris-HCl, pH 7.5, 2mM EDTA, and 0.1% Triton x-100) at 40°C and washed with the same buffer for 30 min at room temperature. The membrane was incubated in wash buffer containing anti-his antibody (Cell Signaling, 1:500 dilution) overnight and washed three times with Tris-saline buffer (20 mM Tris HCl, 37 mM NaCl, pH 7.5) for 5 min at room temperature. Membranes were then incubated with peroxidase-labeled goat anti-rabbit IgG (Sigma A0545, 1:1,000 dilution) for 90 min. After 3 times wash in Tris-saline buffer, membranes were visualized using ECL and exposed to the X-ray film (Fugi Film). The intensity of bands in the film were analyzed using the Image analyzer (Alpha Innotech FluorChem FC2 MultiImage II).

Results and Discussion

Cloning of the hG-CSF coding DNA sequence

The plasmid contain hG-CSF coding sequence was PCR-amplified with hG-CSF cDNA clone (ATCC 10468871) as a template DNA and primers JA6 and JB6. The 650 bp DNA fragment containing cDNA for hG-CSF obtained after PCR-amplification and restriction enzyme digestion was cloned into the pIZTv5/his vector, an expression vector for a silkworm cell line. The final vector pGCSF codes for his-tagged hG-CSF. The silkworm-specific ER-signal sequences were searched and selected from three extracellular enzymes of silkworm (Fig. 2). The signal sequences of PPAE, PDI, and BX were fuzed to the N-terminal of the mature human G-CSF and the proper signal sequences were determined after signal peptide prediction analysis using SignalP 3.0 Server (Fig. 3).

The ER-signal sequence of hG-CSF was replaced with that of silkworm enzyme PPAE by two-step PCR. Mature human G-CSF coding sequence was amplified first with pGCSF as a template, the primer JA6F, and the reverse primer JB6. The

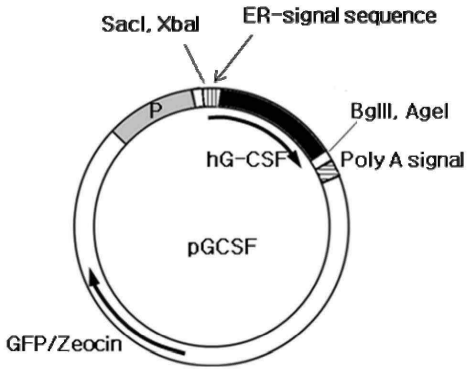

Fig. 1. The plasmid map of phG-CSF. The plasmid encodes for the human G-CSF precursor protein gene. P in grey box represents OplE2 promoter.

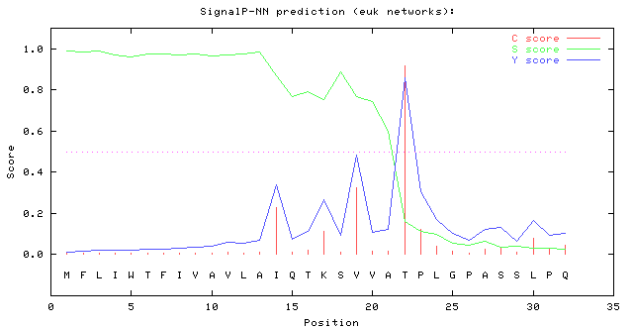

Fig. 2. Signal sequence prediction analysis. Amino acid sequence of G-CSF fused with signal peptide of prophenoloxidase activating enzyme was analyzed using SignalP 3.0 server. The program predicted cleavage site between amino acid A21 and T22.

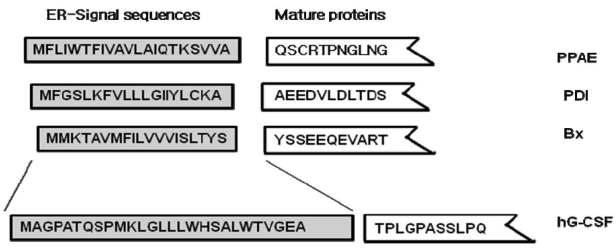

Fig. 3. Replacement of ER-Signal sequence of hG-CSF with ER-Signal sequences derived from silkworm secretory proteins. Sequences in grey boxes and white boxes represent amino acid sequences of ER-signal peptides and mature proteins, respectively. Each ER-signal sequence of silkworm proteins was replaced with that of hG-CSF. PPAE: prophenoloxidase activating enzyme, PDI: protein disulfide isomerase, Bx: bombyxin.

coding region of hG-CSF with PPAE signal sequence was then amplified with the first PCR reaction as a template and the primers JA4F and JB6. The DNA fragment was digested

with SacI and AgeI and cloned into the pIZTv5/his vector cut with the same set to construct pPPAE_GCSF. The expression plasmids, pPDI_GCSF and pBX_GCSF coding for hG-CSF gene including silkworm signal sequences PDI and BX were generated similarly.

Expression and detection of rhG-CSF

The hG-CSF expression plasmids, pGCSF (Fig. 2), pPPAE_GCSF, pPDX_GCSF, and pBX_GCSF, were transfected to BM5 cells and collected culture supernatant four day post transfection. The secreted rhG-CSF in the culture medium was analyzed by Western blot analysis. The rhG-CSF was successfully expressed and secreted in the culture medium of BM5 cells transfected with the expression plasmids with coding sequences for hG-CSF with silkworm signal peptides, PPAE, PDI and BX, while wild type hG-CSF was not detected in the culture medium transfected with plasmid pGCSF (Fig. 4). This result demonstrated that the hG-CSFs with ER-signal peptides of silkworm proteins, PPAE, PDI, and BX, are responsible for the efficient production of rhG-CSF in the silkworm cell line BM5.

The time course of rhG-CSF production

The production of rhG-CSF in the culture medium was detected every day for 7days after transfection of plasmid pPPAE_GCSF to the BM5 cells. The production of the protein reached maximum level at 4 days post-transfection and lasted up to 7 days (Fig. 5).

Secretion of the expressed recombinant protein in the culture medium by the cells transfected with the expression plasmids is advantageous for the protein purification process using a single chromatographic step. The replacement

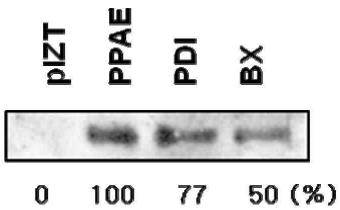

Fig. 4. Expression of recombinant hG-CSF with various silkworm signal sequences. The plasmid expressing hG-CSF and plasmids encoding mature hG-CSF fused with different signal peptides (PPAE, PDI, BX) were transfected. Recombinant hG-CSFs secreted in the culture media were analyzed by western blot using ant-his antibody. The numbers below each band are relative band intensity, which indicated efficiency of each ER-signal sequence.

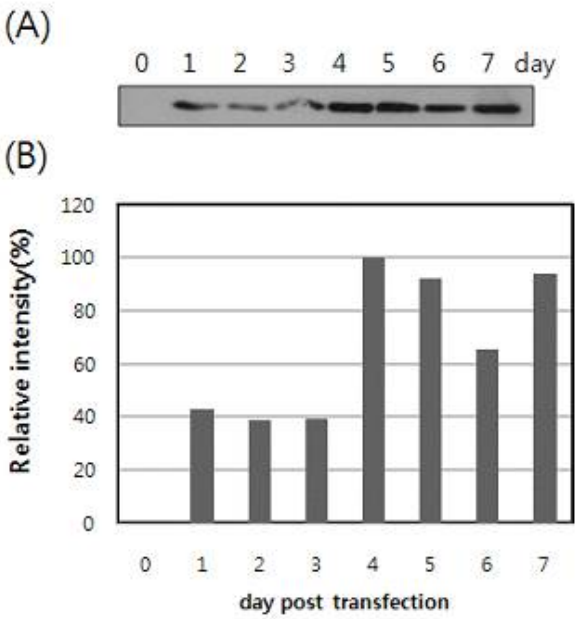

Fig. 5. Production of rhGCSF with ER-signal peptide from PPAE of *Bombyx mori*. (A) The plasmid pPPAE_GCSF was transfected to BM5 cells and culture supernatant was harvested every day for a week. Two ml of each collected medium was analyzed for rhG-CSF expression by western blot using anti-his antibody. The numbers (0-7) indicate days of post-transcription. (B) The protein band intensity in panel A was quantified and expressed as relative percentage.

of ER-signal peptide of hG-CSF with silkworm-specific sequences greatly improved the secretion of rhG-CSF in a culture medium of BM5 cells transfected with the expression plasmids. The rhG-CSF produced in the cell culture medium remained for several days after maximum production implicate that the rhG-CSF is fairly stable for several days after its release into the culture medium. The data obtained here suggest that the human genes need to be modified to achieve efficient secretion of human origin proteins in an insect system.

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초록 : 누에세포를 이용한 인간 G-CSF의 발현 및 생산

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조혈촉진 cytokine인 Granulocyte colony stimulating factor (G-CSF)는 골수세포를 자극하여 granulocyte로 증식, 분화시키는 기능을 가지며, 현재 아주 고가의 치료제로 사용되고 있다. 인간 G-CSF (hG-CSF)를 아직 시도되지 않은 누에 유래 세포주인 BM5 세포에서 발현시키고 생산 효율을 높이기 위해 hG-CSF cDNA를 변형하였다. hG-CSF의 cDNA의 endoplasmic reticulum (ER) signal sequence 부분을 누에의 소포체에서 분비되는 단백질인 prophenoloxidase (PPAE), protein disulfide isomerase (PDI)와 bombyxin (BX)에서 유래한 누에특이 ER signal sequence로 대체한 hG-CSF의 cDNA 함유 벡터를 구축하였다. 이들 벡터를 사용하여 형질전환한 BM5 세포의 배양액에 분비된 G-CSF 단백질을 western blot으로 분석하여 발현을 확인하였다. 누에특이 ER signal sequence들로 대체된 hG-CSF cDNA를 포함하는 벡터에 의한 hG-CSF 단백질 생산이 인간 G-CSF cDNA가 든 벡터에 의한 hG-CSF의 생산보다 월등히 효율적이었다. 또한, PPAE-signal sequence를 포함하는 hG-CSF 단백질은 배양배지에서 형질전환 4일 후에 최고에 달하였고, 7 일째까지 비슷한 양이 배지 내에서 검출되었다. 이상의 결과는 인간유래 유전자가 곤충세포 내에서 발현 될 때 인간유래 유전자 보다는 곤충 유전자발현 시스템에 맞게 변형했을 경우 더 효율적인 단백질 발현을 얻을 수 있음을 보여 준다.