Journal of Life Science 2008 Vol. 18. No. 7. 912~917 ©JLS / ISSN 1225-9918

Biological Activity of Recombinant Human Granulocyte Colony-Stimulating Factor and Isolation of the Somatic Cell Transfected EGFP-hG-CSF Gene

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Received May 1, 2008 / Accepted June 17, 2008

To investigate the biological activity of recombinant human granulocyte colony-stimulating factor (rec-hG-CSF) in mammalian cells, hG-CSF gene was cloned using the cDNA extracted from the human squamous carcinoma cell lines and rec-hG-CSF was produced in CHO cell lines. To analyze the biological activity *in vivo*, the rec-hG-CSF protein was injected into mice subcutaneously on days 0 and 2. Blood was withdrawn for white blood cell (WBC) determination 5 days after the first injection. WBC values were found to have increased significantly. A pEGFP-mUII-hG-CSF vector was transfected into somatic cell lines isolated from bovine fetal cells. The colony expressing EGFP signals was observed with a confocal microscope. These data suggest that the rec-hG-CSF produced in this study has potent activity *in vivo*. Thus, the results of this biological activity show that rec-hG-CSF can be enhanced considerably by genetic engineering that affects potential activity, including mutations, which add the oligosaccharide chain and construct double-fusion proteins. A pEGFP-mUII-hG-CSF vector can be utilized for the production of cloned transgenic livestock.

Key words : G-CSF, recombinant, biological activity, pEGFP-mUII-hG-CSF vector

Introduction

A range of extracellular stimuli, including a network of hematopoietic growth factors and cytokines, regulates the production of blood cells. One of these, granulocyte colony-stimulating factor (G-CSF), is a major regulator of neutrophilic granulocyte production and augments the proliferation, survival, maturation, and functional activation of cells of the granulocytic lineage [2,12]. Severe congenital neutropenia is a rare disease diagnosed at or soon after birth, characterized by a myeloid maturation arrest in the bone marrow, ineffective neutrophil production, and recurrent infections [3].

Neutrophil production is critically regulated by G-CSF and its cognate receptor (G-CSFR). hG-CSF cDNA has been isolated from a cDNA library constructed with mRNA prepared from human squamous carcinoma cells, which produce G-CSF constitutively. G-CSF possesses two alternative splice variants that differ by three amino-acid deletions. Each of these forms of G-CSF is active, although the larger splice form is reported to have a lower specific activity [17,18]. The dexamethasone dose increases correspondingly with the G-CSF levels in healthy men, an effect which may account for some of its effects on neutrophils [5]. Mice G-CSF was generated by targeted disruption of the G-CSF gene in embryonic stem cells. G-CSF-deficient mice (genotype G-CSF-/-) are viable, fertile, and superficially healthy, but have chronic neutropenia. G-CSF is indispensible for maintaining the normal quantitative balance of neutrophil production during "steady-state" granulopoiesis *in vivo* and is implicated G-CSF in "emergency" granulopoiesis during infections [12]. Recently, Druhan *et al.*, [3] reported a novel mechanism of G-CSF refractoriness in patients with severe congenital neutropenia. Extracellular mutations in the G-CSFR in patients with this disease, which is unresponsive to G-CSF, appear to have a common mechanism underlying G-CSF refractoriness.

Several recombinant proteins having *in vivo* biological activity have been identified in our lab [15,16,19].

In the present study, the biological activity of recombinant hG-CSF was determined by the measuring the WBC value after *in vivo* injections. Additionally, the constructed transgenic vector for somatic cell cloning was transfected and the cell lines expressing EGFP-hG-CSF were identified by confocal microscope.

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Materials and methods

Materials

The expression vector pcDNA3 was purchased from Invitrogen Life Technologies (CA, USA). CHO-K1 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Ham's F-12, CHO-S-SFM II, G418 and Lipofectamine were from Gibco BRL (MD, USA). Fetal bovine serum was from Hyclone Laboratories (Utah, USA). The RT-PCR kit and endonucleases were purchased from Takara Korea Biomedical Inc. (Seoul, Korea) and Boehringer Mannheim (MA, USA). The PCR primers synthesized from CoreBio System (Seoul, Korea). Human squamous carcinoma cell line (HS-5; ATCC No: CRL-11882) was from ATCC (USA). The QIAprep-spin plasmid kit was from QIAGEN Inc. (Hilden, Germany), and the hG-CSF Immunoassay kit was from BIOSource International Inc. (CA, USA). All of the other reagents used were from Wako Pure Chemicals (Osaka, Japan) and Sigma Aldrich (MO, USA).

Construction of hG-CSF transfer vector

The primers [sense: 5'-TAg gTA CCA Tgg CTg gAC CTg CCA CCC Ag-3' and antisense: 5'-TCC TCg AgT Cag ggC Tgg gCA Agg Tgg CgT Ag-3'] were designed from the previously reported nucleotide sequence of hG-CSF cDNA [17]. Total RNA was extracted from the CRL-11882 cancer cell lines expressing the hG-CSF protein. A PCR template was used with the cDNA of this cell lines. PCR fragments were ligated into pCR2.1 vector and sequenced completely to confirm the Kozak site and PCR errors. After digestion with Kpn I and Xho I, the fragments were inserted into the Kpn I and Sal I sites of pcDNA3.

Cell culture and functional expression

The expression vector (pcDNA3) was transfected into CHO-K1 cells by the liposome formulation (Lipofectamine) transfection method according to the directions of the supplier. rec-hG-CSF protein produced via the transient method was collected from the supernatants 72 hr after transfection. Stable cell transfectants were selected by incubation in a growth medium [Ham's F12 media containing penicillin (50 U/ml), streptomycin (50 mg/ml), glutamine (2 mM) and 10% FCS] supplemented with G418 (800 ug/ml) for 2-3 weeks post-transfection according to a previously reported method [15]. After incubation of selected stable cells (1×10^6) in 20 ml CHO-S-SFM-II at 37°C for 48 h, the culture media were collected and centrifuged at 14,000 rpm, at 4°C for 60 min to remove the cell debris. The amount of recombinant hG-CSF was quantified using the ELISA method according to the protocol of the supplier (BIOSource International Inc. CA, USA).

In vivo biological activity

Two groups of mice (ICR) were injected subcutaneously with 60 pg/ml of rec-hG-CSF on days 0 and 2. Blood was withdrawn for WBC determination. WBC values were measured 5 days after the first injection.

Construction of transgenic vector (EGFP-hG-CSF) for somatic cell cloning

Initially, mouse uroplakin II promoter (3,603 bp) from the mouse bladder genome was amplified by PCR (sense primer: 5'-gAA TTC CTC gAC gAT CTC ggC CCT CTT TCT gC-3'; antisense primer: 5'-CCA ggA TCC AgT CCC AgC gCA gTg gTA CC-3') and was cloned into a pCR 2.1 vector. The primers were inserted the Eco *RI* and Kpn *I* sites.

The mouse UII promoter cloned into the pCR 2.1 vector was cut using Kpn *I* and Hind *III* enzymes. SV40 (2.6 kb) was then amplified with sense primer (Kpn *I*+Sal *I* site addition) and antisense primer (Eco *RV*+Hind *III* site addition). A PCR product was cut using Kpn *I* and Hind *III* enzymes and was then introduced into the same sites of mUII promoter cloned into the pCR 2.1 vector (pmUII-SV40). The pmUII-SV40 was digested with Kpn *I* and Sal *I*, and hG-CSF cDNA was then combined for the construction of the expressing vector (pmUII-hG-CSF).

In order to add the GFP gene, the EGFP gene (749 bp) of pEGFP-C2 vector was amplified by PCR primers (sense primer: 5'-TCA gAT CCg CTA gCg CTA CCg gTC-3'; antisense primer: 5'-ggg CCC TTA ACT TgT ACA gCT CgT CCA T-3') with Nhe *I* and Apa *I* sites.

The EGFP gene was cloned into the pCR 2.1 Vector and sequenced to determine PCR errors. Nhe *I* and Apa *I* fragments of EGFP were introduced into the same sites of pcDNA3.1/Zeo. 1,697 bp DNA containing CMV promoter, BGH poly A and EGFP DNA, were amplified with Not *I* site primer (5'-gCg gCC gCg Atg TAC ggg CCA gAT ATA C-3') and Eco *RI* site primer (5'-gAA TTC TCC CCA gCA TgC CTg CTA TT-3'). Finally, the fragment and pmUII-hG-CSF were digested with Not *I* - Eco *RI* enzymes and were combined for the construction of the transgenic

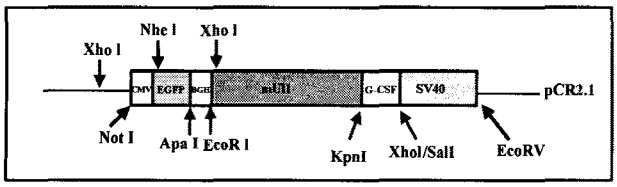

Fig. 1. Diagram of vector encoding the EGFP and hG-CSF. The expression vector (pEGFP-mUII-hG-CSF) comprises a promoter based on the mouse uroplakin II and SV40 poly A, as described in the *Materials and Methods* section.

vector for somatic cell cloning (pEGFP-mUII-hG-CSF). The direction of the expression transfer was confirmed by restriction mapping (Fig. 1).

Somatic cell transfection and expression of GFP

Bovine fetal fibroblast cells were isolated from a 45-day-old fetus recovered surgically from a Korean native cow. Transfection of the pEGFP-mUII-hG-CSF was done according to a previously reported method [15]. The signal of GFP expression was recorded with a confocal microscope (Fluoview, Olympus, Japan).

Results

Cloning of hG-CSF cDNA and mouse uroplankin II promoter

PCR was performed using the primers designed from sequences that were published previously (Fig. 2 left). A cDNA prepared from CRL-11882 cell lines. The cDNA fragment with 624 bp predicted for hG-CSF cDNA was amplified. The fragment was subcloned into the pCR2.1 cloning vector and sequenced. An analysis of the nucleotide sequence of the hG-CSF showed that it was identical to data reported previously. Mouse uroplakin II promoter (3,603 bp) from the mouse bladder genome was then amplified. It was cloned into the pCR 2.1 vector (Fig. 2 right). The sequence result was the same nucleotide as previously reported. The expressing vectors were then constructed for utilization in CHO cell lines (pcDNA3-hG-CSF) and somatic cells (pEGFP-mUII-hG-CSF).

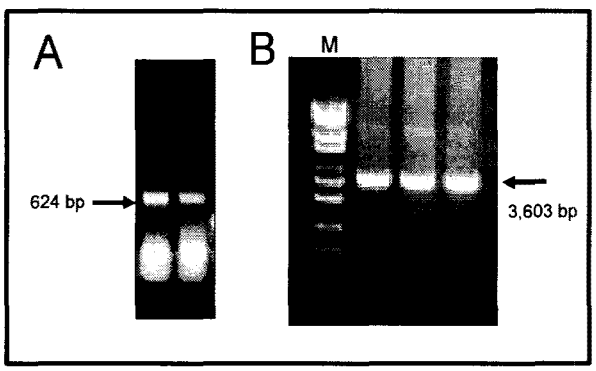

Fig. 2. PCR amplification of hG-CSF cDNA and mUII promoter. The PCR products predicted with each primer were analyzed by 1.0% agarose electrophoresis. The major fragments, which were 624 bp (hG-SCF cDNA: A) and 3,603 bp (mUII promoter: B), were amplified.

Production of transient and stably expressing hG-CSF

G-CSF cDNA vectors were transfected into CHO-K1 cells. Transient expression was collected in the supernatants 72 hr after transfection. Between six and eight clones of stably transfected pools were selected for G-418. After incubation of the selected stable cells (1×10^6) in 20 ml CHO-S-SFM-II at 37°C for 48 hr, the culture media were collected and centrifuged at 15,000 rpm, at a temperature of 4°C for 60 min to remove the cell debris. The clonal cell lines isolated with G418 were subjected to reverse transcription-PCR (Fig. 3). The amount of rec-hG-CSF was quantified using ELISA (Fig. 4). The transient expressing quantity was 28 to 32 pg/ml (Fig. 4 left: cell clone number 1 and 2). However, quantity of stable cell lines was less than 10 pg/ml (Fig. 4 left: cell clone number 4 to 6). Thus, the quantity of rec-hG-CSF was concentrated and ELISA was used to measure the result, which ranged from 40 to 80 pg/ml. Thus, the rec-hG-CSF was concentrated nearly 8 times (Fig. 4 right: cell clone number 1 to 4).

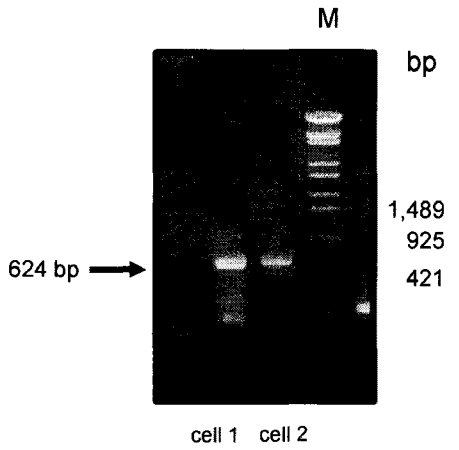

Fig. 3. RT-PCR analysis for hG-CSF mRNA. Total RNA was extracted from two cell lines. The stable cell lines isolated by G418 were subjected to RT-PCR. It was then detected by UV spectrophotometer.

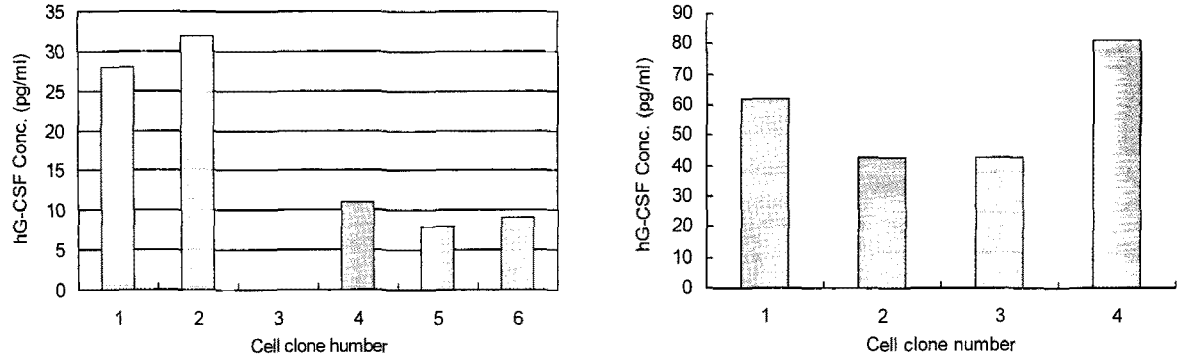

Fig. 4. Transient and stable expression of hG-CSF cDNA. The transient expression of hG-CSF is shown (left; number 1-2). The stable expression is also shown (left; number 4-6). The supernatants were concentrated several-fold using an Amicon Centriplus device (5,000 rpm at 4°C) and were then subjected to ELISA. Experiments were performed in triplicate.

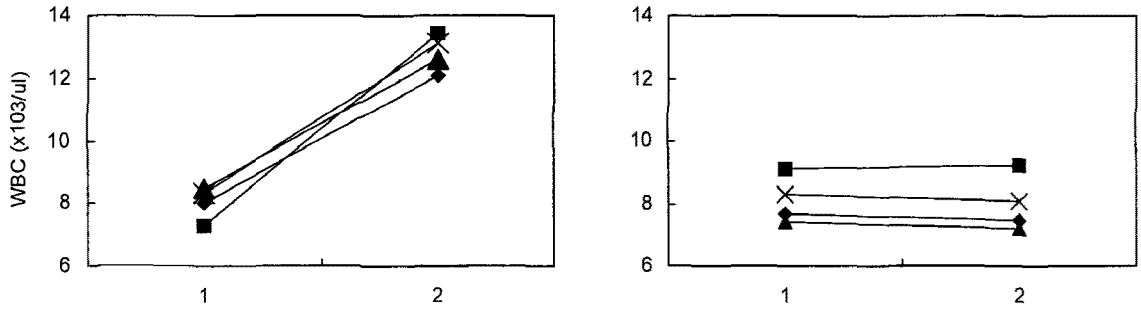

Fig. 5. Rec-hG-CSF induces an increase of WBC in mice. Two groups of ICR mice were injected subcutaneously with 60 pg of rec-hG-CSF on days 0 and 2. WBC was determined pretreatment day and 5 days after the injection. Values are given for each mouse (left: rec-hG-CSF; right: control).

In vivo biological activity

Two groups of mice were injected subcutaneously with 60 pg/ml of rec-hG-CSF on days 0 and 2. WBC values measured 5 days after the first injection. Mean WBC values were found to have increased significantly from 8.0 to 12.8 ($\times 10^3$/ul) (Fig. 5). The values of negative control groups did not increase. The other composition of the blood was not changed (data not shown). The considerable increase was coursed by the administration of rec-hG-CSF. The rate of increase was nearly 50% compared with pretreatment conditions. Thus, rec-hG-CSF could increase the WBC value.

Somatic cell transfection and expression of GFP

The transfection for somatic cells utilized a method involving liposome-mediation with the EGFP+mUII+hG-CSF gene. The result showed a high number of colonies. A GFP signal was detected with a confocal microscope (Fig. 6 left), which was then magnified 4X (Fig. 6 right). EGFP cell lines expressing fluorescent protein were selected and a single clone was isolated. Each cell line was established

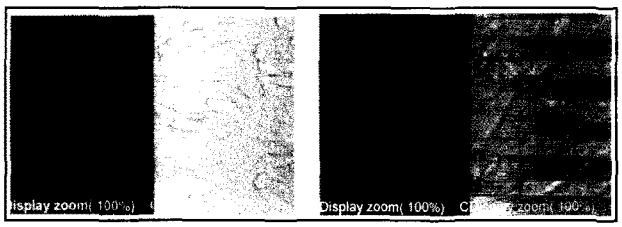

Fig. 6. Confocal micrographs of a somatic cell transfected with pEGFP-mUII-hG-CSF vector. A somatic cell transfected with pEGFP-mUII-hG-CSF vector was recorded with a confocal microscope (Fluoview). The right panel is a magnified image.

and well stocked in LN2 tank. The vector used in this study could be selected easily for the expression of EGFP in the transfected somatic cell.

Discussion

In the present study, it was shown that rec-hG-CSF produced in the CHO cell lines has enhanced biological activity *in vivo*. A pEGFP-mUII-hG-CSF vector was constructed and the somatic cell lines expressing these genes were

isolated. Thus, the vector could easily select the somatic cell line to produce cloned the transgenic livestock. Mice were injected subcutaneously with 60 pg/ml of rec-hG-CSF on days 0 and 2. Mean WBC values were shown to increase significantly from 8.0 to 12.8 ($\times 10^3$/ul). These data suggest that the rec-hG-CSF produced in this study has potent activity *in vivo*.

Administration of purified rec-hG-CSF was reported to significantly increase the number of neutrophils significantly in patients with carcinomas who were undergoing chemotherapy and in patients with AIDS and neutropenia [13,14]. Kaneko *et al.* [6] reported marked improvement after the administration of rec-hG-SCF to a patient with granular proliferative disease. G-CSF was reported to promote survival of cardiac myocytes [4]. Lehrke *et al.* [11] reported that G-CSF lacks therapeutic efficacy in older animals. G-CSF augmented stem cell filtration without increasing the number of inflammatory cells [1]. G-CSF, alone or in combination with stem cell factors can improve the hemodynamic cardiac function after myocardial infarction [9]. G-CSF and stem cell factor therapy improved the cardiac function when delivered after myocardial infarction by increasing the number of blood vessels and cells of cardiomyogenic lineage [7].

G-CSF forms a tetrameric complex with its receptor, comprising two G-CSF and two receptor molecules, indicating that Glu^{19} of G-CSF interacts with Arg^{288} of the receptor [10]. Ward *et al.* [20] identified the membrane-distal domain of the G-CSF receptor as being for the transduction of differentiation signals. The cytoplasmic tail of the G-CSF receptor was truncated in a subset of patients with severe congenital neutropenia transforming to acute myeloid leukemia [3].

Transgenic mice expressing hG-CSF in their urine was previously reported by the authors [8]. The hG-CSF was secreted into urine at a high level (approximately 500 pg/ml) and was able to enhance the proliferation of DMSO-treated HL-60 cells, suggesting that the transgenic urine-derived hG-CSF was bioactive [8]. The bladder is an attractive organ of choice for the production of pharmaceuticals as urine is easily collected during the lifetime of a transgenic animal irrespective of sex. In addition, due to the highly efficient nature of secretion via urine, the urinary tract is considered to be a useful system to produce biological materials that might cause deleterious effects when they accumulate and/or circulate inside the blood system of a transgenic animal.

In this study, a specific vector expressing the EGFP gene was constructed. The mouse uroplakin II promoter was utilized to evaluate the feasibility of using an animal bladder for the production of biologically active proteins. The results are presented regarding the combination of the information gained from both genetic and biochemical approaches. Recombinant G-CSFs including mutations, that change oligosaccharides, will be useful tools for analyzing the structure-function relationships. New analogs can also be constructed to include additional specific bioactive generating potentially efficacious treatment.

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초록 : 유전자 재조합 인간의 G-CSF의 생리활성과 EGFP-hG-CSF유전자가 도입된 체세포의 분리

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유전자재조합 hG-CSF의 생리활성을 분석하기 위하여 편상의 암세포로부터 분리되어진 cDNA를 이용하여 hG-CSF 유전자를 분리하여 동물세포(CHO cell lines)를 이용하여 재조합 단백질을 생산하였다. 재조합 단백질의 체내 생리활성을 분석하기 위하여0일과 2일에 피하주사 후 5일에 혈액을 채취하여 백혈구 수를 분석하였다. 투여 전과 비교하여 5일째에 백혈구 수는 현저하게 증가하였다. 또한, pEGFP-mUII-hG-CSF벡터를 소 태아로부터 분리되어진 체세포에 형질전환을 시켜서, EGFP signal을 나타내는 세포를 confocal를 이용하여 분리하여 수립하였다. 따라서, 이러한 결과는 유전자재조합 hG-CSF는 체내에서 강력한 생리활성을 나타내며, 또한 당쇄가 첨가되어지고 이중으로 연결되어진 새로운 돌연변이체를 포함하여 고 활성 재조합체의 생산이 가능할 것으로 보이며, pEGFP-mUII-hG-CSF벡터는 복제 형질전환 가축 생산을 위하여 유용하게 사용되어질 것으로 사료된다.