

Production of Recombinant Human Granulocyte Macrophage Colony-Stimulating Factor from Silkworm *Bombyx mori* Bm5 Cells

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Using silkworm *Bombyx mori* Bm5 cells, we established a stable cell line expressing the human granulocyte macrophage colony-stimulating factor (hGM-CSF), which gets its name from the Bm5-hGM-CSF cell in which the glycoprotein of the hGM-CSF is secreted in the cell culture supernatant (CCS). It was demonstrated that secreted hGM-CSF had *in vivo* biological activity and the white blood cell (WBC) value increased two times that of the control. We expect to produce useful human recombinant glycoproteins from silkworm cultured cells for a low price and a large quantity.

Key words: Silkworm *Bombyx mori* Bm5 Cells, Human Granulocyte Macrophage Colony-Stimulating Factor, White Blood Cell

Introduction

Various mammalian culture cell lines may be particularly useful for the expression of recombinant glycoproteins due to their capacity to perform correct posttranslational modifications in the endoplasmic reticulum (ER). Thus, they are routinely used for the production of commercially valuable recombinant protein materials. However, the cost of human hormones used in the production of recombinant proteins using mammalian cell lines is a major problem (Jenkins *et al.*, 2009).

The insect cell of silkworm *Bombyx mori*, Bm5, has an ER in which mammalian-like posttrans-

lational modification processes occur, giving it merit as a potentially valuable tool for low-cost glycoprotein mass-production using no expensive hormones and/or growth factors. So far, although the baculovirus-expression system using Bm5 cells is successfully used to produce recombinant proteins, production of useful biomaterials directly from Bm5 cells has some advantages, including a need to not inactivate baculovirus itself, and no rejection of consumers of useful biomaterials produced by baculovirus (Hitchman *et al.*, 2009).

The human granulocyte macrophage colony-stimulating factor (hGM-CSF) is a glycoprotein produced by mesenchymal cells, which plays a crucial role in macrophage differentiation and proliferation. In addition, it also is reported that the hGM-CSF stimulates the production and antibacterial function of neutrophils and monocytes (Metcalf, 1985). Recently, the hGM-CSF has been used as a medication to stimulate the production of white blood cells (WBCs) for chemotherapy and has been evaluated in clinical trials for its potential as a vaccine adjuvant in HIV-infected patients (Kedzierska *et al.*, 1998; Conti and Gessani, 2008). Following a number of clinical demands, recombinant hGM-CSF was produced in many organisms, including microbes, yeast, mammalian, and plant cells. However, these organisms have wick points for producing useful human-type glycoproteins.

Here, for the first time, we establish a transgenic Bm5 (Bm5-hGM-CSF) cell that constantly secretes hGM-CSF as a glycoprotein type with bioactivity for proliferation of WBCs *in vivo*.

Material and Methods

Bm5 cells (silkworm *Bombyx mori* cell line derived from ovary) were cultured in Grace's insect medium (WelGENE, Daegu, Korea), including 10% FBS (fetal bovine serum) and 1% antibiotic-antimycotics solution (Sigma-Aldrich, St. Louis, USA), at 26 °C (Goo *et al.*, 2004). A cDNA encoding hGM-CSF was cloned into the *Kpn* I and *Eco*R I sites of the insect expression vector pIZT/V5-His (containing green fluorescent protein as a screening marker). The resulting vector was transfected into Bm5 cells using lipofectin reagent (Invitrogen, Carlsbad, USA). Transfected cells were cultured under 200 µg/ml antibiotic Ze-

ocin (Invitrogen) for 14 d. Successfully transfected cells (Bm5-hGM-CSF) were screened by RT-PCR and for green fluorescence under a fluorescence microscope. Total RNA from cultured cells was extracted using an RNA isolation reagent (TRI reagent; Ambion, Austin, USA). RT-PCR using the forward primer (F) 5'-ACCACCAGTC-CATCGCCATT-3' and the reverse primer (R) 5'-CCACCCTGGACGGAAGTTTG-3' for the hGM-CSF was performed for 27 cycles under the following conditions: 94 °C for 30 s, 57 °C for 40 s, and 72 °C for 40 s (but 10 min in the final cycle) with *Taq* DNA polymerase. Immunoblotting analysis was performed according to standard procedures. Bm5-hGM-CSF cells were scraped, lysed by addition of SDS sample buffer [62.5 mM Tris-HCl, pH 6.8, 6% (w/v) SDS, 30% glycerol, 125 mM DTT, 0.03% (w/v) bromophenol blue], and separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, which was then incubated with the primary antibody (recognizing His tag) overnight at 4 °C. Blots were developed using the kit for the ECL Western blotting detection system (GE Healthcare, Piscataway, USA) and BioMax Light film (Kodak, Rochester, USA). ELISA (enzyme-linked immunosorbent assay) was performed using the hGM-CSF ELISA kit (KOMA Biotech, Seoul, Korea) in 96-well culture plates. After treatment with the coating antibody recognized by hGM-CSF for 2 h at room temperature, cell culture supernatant (CCS) containing secreted hGM-CSF was added and reacted. After 2 h at room temperature and washing with PBST (0.1% Tween 20 in PBS), horseradish-peroxidase (30 min reaction) and TMB solution (10 min reaction) were added. When light was emitted, 2 M H₂SO₄ was added, and the 96-well culture plate was estimated at 450 nm by a microplate reader. CCS containing the secretory form of recombinant hGM-CSF was injected into mice (Balb/c) intraperitoneally. Blood was collected for WBC determination 4 d after the first injection. WBCs were estimated using an automated blood cell analyzer (Siemens Medical Solutions Diagnostics, Tarrytown, USA). Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich.

Results and Discussion

Bm5 cells were transfected with the modified pIZT/V5-His vector containing genes of both green fluorescent protein (GFP) and hGM-CSF,

for easy observation of the expression of the transfectant with a fluorescence microscope. For selection of cells with stable expression of the gene of the hGM-CSF, Zeocin was added at a concentration of 200 µg/ml 12 h post transfection. The GFP was observed 5 d after transfection. By that time Bm5 control cells were detached from the culture plate. After 14 d of transfection, RT-PCR confirmed a transfectant containing the hGM-CSF. Successfully transfected Bm5 cells were coined Bm5-hGM-CSF cells. As shown in Fig. 1A, hGM-CSF 434 bp was detected in Zeocin-resistant cell clusters, which eliminate the GFP.

Although many kinds of cDNA can be transfected into a cultured cell line, it is not always expressed by its mRNA and protein. In this study, it was confirmed that a cDNA of the hGM-CSF was transfected into Bm5 cells, and transcription and translation were successfully demonstrated by checking both intracellular and secretory hGM-CSF. Fig. 1B shows results from Western blotting; lane 1 is the cell lysate used as a control. Lane 2, however, is used as the cell lysate of the Bm5-hGM-CSF cell. The 22-kDa hGM-CSF was only detected in the cell lysate of the Bm5-hGM-CSF cell, which means that a transfected cDNA of the hGM-CSF is successfully processed to transcription and translation in Bm5 cells. Next, we checked whether the hGM-CSF in a cell is correctly secreted into the extracellular environment. Lanes 3 and 4 in Fig. 1B also show the result of Western blotting; lane 3 is the collected cell CCS used as a control, and lane 4 is the collected CCS from Bm5-hGM-CSF cells. An obvious level of the hGM-CSF is shown only in lane 4. As shown in Fig. 1B, these results demonstrate that exogenous hGM-CSF cDNA is successfully transcribed and translated (lane 2), and secreted (lane 4) from Bm5-hGM-CSF cells.

We next sought to confirm whether or not the hGM-CSF from the Bm5-hGM-CSF cell is a glycoprotein. Bm5-hGM-CSF cells were treated with tunicamycin, which inhibits *N*-linked glycosylation of glycoprotein. After the culture medium was exchanged, the same volume of CCS was collected from Bm5-hGM-CSF cells at indicated times, with or without tunicamycin. Fig. 1C shows the result of Western blotting using His-primary antibody. Mis-/malformed hGM-CSF by inhibition of *N*-linked glycosylation is not secreted from Bm5-hGM-CSF cells, but is retained rather in the lumen of the ER; however, newly synthesized

hGM-CSF produced without tunicamycin is successfully secreted. Thus, we can confirm that the hGM-CSF produced from Bm5-hGM-CSF cells (secretory form indicated by an arrow) is a glycoprotein successfully secreted from the ER to the extracellular environment.

The highest level of hGM-CSF production was detected by Western blotting 48 h following ex-

change of new culture medium (data not shown). At this time, the concentration of the hGM-CSF in the CCS was determined by ELISA. The maximum concentration of extracellular hGM-CSF was 4.2 ng/ml (Fig. 1D).

We next demonstrated whether or not the hGM-CSF in the CCS has bioactivity. One of two groups of mice was injected intraperitoneally with

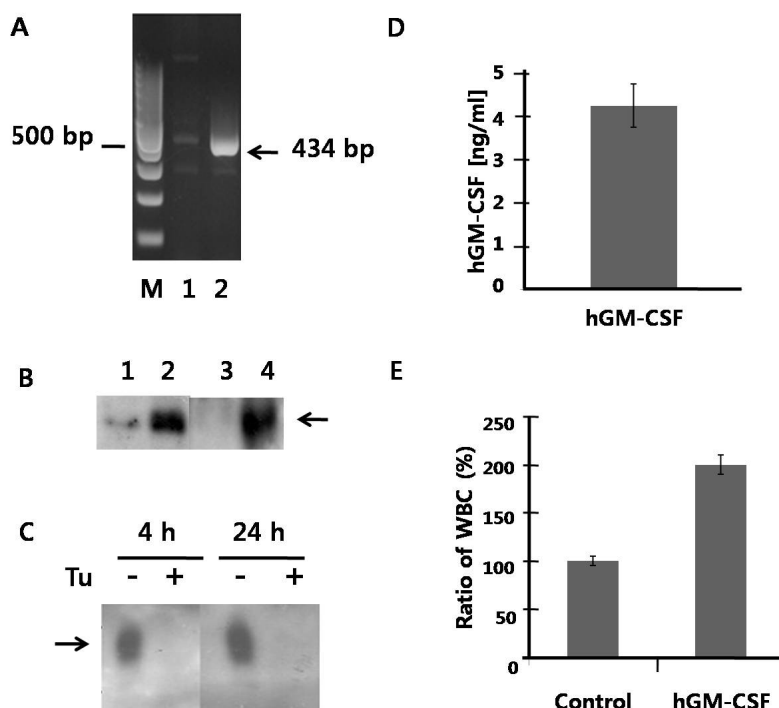


Fig. 1. Production of recombinant hGM-CSF from *Bombyx mori* Bm5 cells. (A) Results of RT-PCR to screen for Bm5-hGM-CSF cells. Bm5 cells were cultured in Grace's insect medium that included 10% FBS and 1% antibiotic-antimycotics solution at 26 °C. The insect expression vector pIZT/V5-His contained hGM-CSF cDNA in the *Kpn* I and *EcoR* I sites. The resulting vector was transfected into Bm5 cells using lipofectin, and transfected cells (Bm5-hGM-CSF cells) were screened under Zeocin (200 µg/ml) for 14 d. The RT-PCR product of 434 bp was detected, corresponding to the full length hGM-CSF (lane 2). Lane 1 is the negative control using non-transfected cells. (B) Western blotting of hGM-CSF. Intracellular hGM-CSF was immunoblotted with antiHis-antibody under conditions of either transfected (lane 2) or not transfected (lane 1) with hGM-CSF cDNA. Using the same method, secretory hGM-CSF was detected under conditions of either transfected (lane 4) or not transfected (lane 3) with hGM-CSF cDNA. (C) Inhibition of hGM-CSF by tunicamycin. Cells were treated with tunicamycin (Tu, 2 µg/ml; indicated by +) or without (–) for the indicated time (4 and 24 h) after which the same volume of CCS was collected in 100 µl of SDS sample buffer. After boiling for 5 min, 15-µl aliquots of each sample were subjected to SDS-PAGE (10% gel) and analyzed by immunoblotting with antiHis-antibody. (D) Determination of the hGM-CSF concentration in CCS by ELISA. ELISA assay processing was performed using the hGM-CSF ELISA kit (KOMA Biotech) following the manufacturer's instructions. The final concentration was estimated at 450 nm by a microplate reader. (E) Bioactivity of hGM-CSF *in vivo*. Collected CCS containing the secretory form of recombinant hGM-CSF (2 ng) was injected into mice (Balb/c) intraperitoneally. Positive control mice were injected with the same volume of PBS. 4 d after injection, mice were killed and WBCs were estimated using an automated blood cell analyzer (Siemens Medical Solutions Diagnostics). Although the experiments were performed in triplicate, only a representative blot is shown in the figures. Mean values from three independent experiments done in duplicate at $p < 0.05$ are presented.

CCS containing 2 ng hGM-CSF. Another group was injected intraperitoneally with Bm5 culture medium (control). WBC values were measured 4 d after injection. The increase was nearly 2-fold higher than that of the control (Fig. 1E). In addition, no morphologically abnormal WBC was detected (data not shown). It is therefore suggested that secreted recombinant hGM-CSF from Bm5 cells has a valuable biological activity for WBC proliferation *in vivo*.

In summary, exogenous human cDNA encoding glycoprotein, hGM-CSF, is successfully biosynthesized through correct transcription and translation, eventually producing the secretory form of

the bioactive glycoprotein in the Bm5 cell (silkworm *B. mori* cell line), due to similar posttranslational modifications with human glycoproteins. Other big advantages of using Bm5 cells are low cost and mass-production of bioactive glycoproteins, with no need for expensive hormones for culture.

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