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Secretory Production of hGM-CSF with a High Specific Biological Activity by Transgenic Plant Cell Suspension Culture

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Abstract The human granulocyte-macrophage colony stimulating factor (hGM-CSF) gene was introduced into tobacco plants. The cell suspension culture was established from leaf-derived calli of the transgenic tobacco plants in order to express and secrete a biologically active hGM-CSF. The recombinant hGM-CSF from the transgenic plant cell culture (prhGM-CSF) was identified as a yield of about 180 μg/L in the culture filtrate, as determined by ELISA. The addition of 0.5 g/L polyvinylpyrrolidone (PVP) to the plant cell culture medium both stabilized the secreted prhGM-CSF and increased the level of production approximately 1.5-fold to 270 μg/L. The biological activity of the prhGM-CSF was confirmed by measuring the proliferation of the hGM-CSF-dependent cell line, TF-1. Interestingly, the specific activity of the prhGM-CSF was estimated to be approximately 2.7 times higher than that of a commercially available preparation from *E. coli*.

Keywords: hGM-CSF, tobacco, plant cell culture, secretion, protein stabilization

INTRODUCTION

Granulocyte-macrophage colony stimulating factor (GM-CSF) was one of the first of large number of cytokines purified and cloned [1]. The mature human GM-CSF (hGM-CSF) contains 127 amino acid residues and is preceded by a hydrophobic leader sequence of 25 amino acid residues in length. The hGM-CSF has increasing clinical applications in the treatment of neutropenia and aplastic anaemia, and has greatly reduced the infection risk associated with bone marrow transplantation by accelerating neutrophil [1]. GM-CSF has been produced in various foreign hosts, such as *Escherichia coli* [2], yeast [3], insect cells [4], *Aspergillus niger* [5] and plant cells [6,7] and is now applied for clinical uses.

Recently, progress in molecular biology has made it possible to utilize transgenic plants as hosts for the recombinant protein expression. Production of valuable proteins through plant cell culture has several advantages over either prokaryotic or animal cell expression systems [8-10]. For example, plant cells are generally inexpensive to grow on a large scale, and their production is not limited to fermentation capabilities. Mmammalian glycoproteins are also glycosylated when they are produced in transgenic plants. However, the *N*-glycan maturation of the recombinant proteins differs between plants and mammals [11,12]. The proteins produced by plant cell culture are also safer than those produced from prokaryotic and animal expression systems, especially for the production of therapeutic proteins applied for the treatment of human diseases [10]. Finally, purification of the target protein is much more economical and easier than with other expression systems because the target protein is secreted into the simple nutrient medium by signal peptides that direct transport of the peptides into the lumen of the endoplasmic reticulum (ER). After their transport into the ER, proteins leaving the ER enter the default secretory pathway to the Golgi apparatus and ultimately to the extracellular space [10,13]. However, there are several limitations to the plant cell culture system, such as slow growth rate and low expression levels, need to be resolved to establish the plant cell culture as an efficient heterologous expression system [14, 15].

In the present study, we report the production of hGM-CSF with high specific biological activity through a genetically engineered plant cell suspension culture. In addition, the effects of the cell culture preservative on the production of the hGM-CSF from plant cell suspension cultures were also studied.

MATERIALS AND METHODS

Expression Vector Construction

The hGM-CSF cDNA was synthesized using RT-PCR

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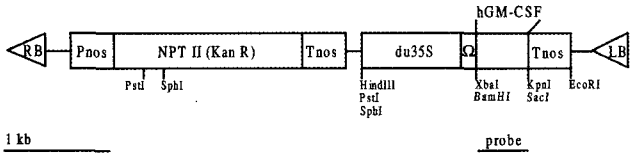

Fig. 1. A schematic diagram of the gene construct utilized in the present study. Transferred-DNA of the final plasmid is shown. RB, LB, Pnos, NPT II, Tnos, du35S, and Ω represent T-DNA right border, T-DNA left border, promoter of nopaline synthase, neomycin phosphotransferase II, terminator of nopaline synthase, CaMV35S promoter with a duplicated enhancer region, and the DNA coding for the mRNA leader sequence, respectively.

technique with poly $(A)^+$ mRNA from PHA and human recombinant IL-12 stimulated peripheral blood mononuclear cells. A pair of primers (5'-GCG GAT CCG TTC TCT GGA GGA TGT-3' and 5'-TTG GTA CCA TCT GGC CGG TCT CA-3') were designed to generate the 471bp PCR fragment containing the open reading frame of the hGM-CSF gene with its own signal peptides [16]. The PCR product was cloned into the pGEM-T vector (Promega, WI, USA) to generate the pMYO62. The hGM-CSF cDNA in the pMYO62 was digested with *Bam*HI and *Kpn*I, and cloned into the same restriction sites of the pMY27 expression vector, *i.e.* between the CaMV35S promoter with a duplicated enhancer region (-417 to -90) including the Ω DNA sequence from the coat protein gene of the tobacco mosaic virus and the nos terminator. The resultant plasmid pMYO64 (Fig. 1) was transferred from the *E. coli* strain HB101 into the *A. tumefaciens* strain LBA4404 by triparental mating using the *E. coli* strain HB101 containing pRK2013 as the helper [17].

Tobacco Transformation

In order to transform tobacco plants (*Nicotiana tabacum* L. cv Havana SR), an *Agrobacterium*-mediated transformation method was applied [18]. Briefly, a young tobacco leaf disc was surface-sterilized and then co-cultured with *A. tumefaciens* LBA4404 harboring the pMYO64. Following co-cultivation, the explants were transferred to MS medium [19] supplemented with 0.1 mg/L α-naphthaleneacetic acid, 1 mg/L 6-benzylaminopurine, 300 mg/L kanamycin, and 500 mg/L cefotaxin for selection. The explants were then transferred onto fresh medium after 2 to 3 weeks from the time of transfer. The well-developed shoots were transferred to hormone free MS medium containing 500 mg/mL cefotaxin and 300 mg/L kanamycin to induce the roots. Finally, the plantlets were transferred to soil and allowed to grow to maturity in the greenhouse.

Plant Cell Culture

Transgenic suspension cell lines were obtained from the leaf-derived calli of transgenic plants, and the tobacco suspension cells were cultured at 25°C in 50 mL of MS medium containing 2 mg/L 2,4-dichlorophenoxyacetic acid, 0.02 mg/L kinetin, and 3% sucrose using a shaking incubator with a rotation speed of 110 rpm. The suspension cell culture was maintained by transferring a fifth of the proceeding culture to fresh medium every 7 days.

Northern Blot Analysis

Total RNA was isolated from transgenic plants by using the RNeasy plant total RNA mini kit (Qiagen, CA, USA). Northern blot analysis was carried out according to the procedure of Sambrook *et al.* [20]. The blots for PCR-Southern and Northern blot analyses were hybridized with α-^{32}P-labeled random-primed DNA using an approximately 460bp *Bam*HI and *Kpn*I fragments from the pMYO62 that included *hGM-CSF*.

Western Blot analysis

Samples were blotted to PVDF membranes after SDS-PAGE [21]. Rat monoclonal anti-hGM-CSF (Pharmingen Inc. CA, USA) at a 1:200 dilution, and HRP conjugated goat anti-rat IgG (Sigma, MO, USA), at a 1:7,000 dilution were used as the primary and secondary antibodies.

Preparation of Samples

Fifty mL of cell suspension was centrifuged at 200 × g for 3 min to prepare culture supernatant and cell pellets. The culture supernatant was lyophilized, redissolved in 2 mL PBS, dialyzed against PBS overnight at 4°C, and used for the culture. The cell pellets were resuspended with 50 mL PBS, homogenized, and concentrated to 2 mL to prepare the fraction.

Quantitative Analyses and Measurement of the Biological Activity of the prhGM-CSF

The quantitative analyses and the measurement of the biological activity of the prhGM-CSF were serviced by the Bank for Cytokine Research (Chonbuk National University, Chonju, Korea). Briefly, the concentration of the prhGM-CSF in the cultured media were determined by the hGM-CSF specific ELISA kit (Endogene, MA, USA) according to the procedure provided by the manufacturer. In order to determine the biological activity of the prhGM-CSF produced by the plant cell suspension culture, the supporting level of the sample for the growth of the hGM-CSF-dependent TF-1 cells was measured [22]. Briefly, growth-factor-starved 1×10^5 cells were suspended in 100 μL of RPMI medium supplemented with 10% FBS (HyClone Laboratories Inc. UT, USA) and added into each well of a microtitre plate containing each the test samples. After incubation for 48 h at 37°C, 1 μCi of [*methyl*-^{3}H]Thymidine (Amersham Lifescience, NJ, USA) was added into each well and incubated for an additional 16 h at 37°C. The cells were harvested with cell harvester (Inotech, Switzerland) and

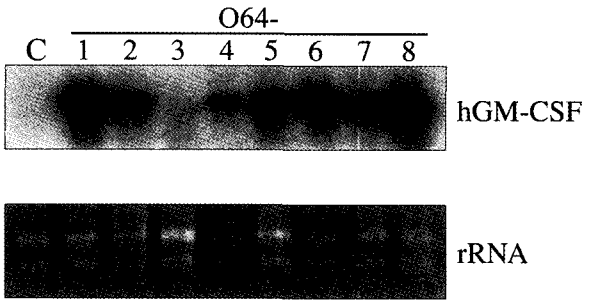

Fig. 2. Northern blot analysis of *hGM-CSF* expression from transgenic plants transformed with pMYO64. Each lane was loaded with 15 μg total RNA from non-transgenic and transgenic plants. Loading standards were indicated by the ethidium bromide stained-rRNA in lower panel. Lane C denotes non-transformant. Lanes 1 to 8 represent the results obtained with the samples prepared from the control plant, O64-1, O64-2, O64-3, O64-4, O64-5, O64-6, O64-7 and O64-8, respectively.

the tritium content was measured with a liquid scintillation counter. In these experiments, a recombinant *E. coli*-derived human GM-CSF, which was purchased from Endogen (MA, USA), was used as a standard.

RESULTS

Vector Construction and Transformation of Tobacco

The cDNA clone of the hGM-CSF was synthesized from PHA and human recombinant IL-12 stimulated peripheral blood mononuclear cells using the RT-PCR technique. The nucleotide sequence of the hGM-CSF was confirmed by DNA sequence analysis using the dideoxynucleotide chain termination method. The hGM-CSF cDNA was cloned into the same restriction sites of the pMY27 expression vector. The *hGM-CSF* was placed under the control of a duplicated CaMV35S promoter including Ω DNA sequence from the coat protein gene of tobacco mosaic virus (Fig. 1). The tobacco leaf discs were transformed with the *A. tumefaciens* strain LBA4404 harboring the pMYO64 plasmid including the neomycin phosphotransferase II (NPTII) gene as a selection marker for the selection of the transformed tissue on kanamycin. The transformed tobacco cells of were selected and regenerated on the media containing 300 mg/L of kanamycin. The presence of *hGM-CSF* in the transgenic tobacco was analyzed using PCR with primers designed to amplify the *hGM-CSF* (data not shown). Finally nine regenerated plants were selected for further analyses.

Northern Blot Analysis

Total RNA from the leaves of the PCR-positive primary transformants was subjected to RNA-blot analysis (Fig. 2). Three *hGM-CSF* transformed plants, namely O64-1, O64-6, and O64-8, expressed very high levels of *hGM-CSF* transcripts, whereas others expressed quite low levels of *hGM-CSF* transcripts. Again, no hybridization was found in the sample prepared from the non-transgenic plant. These results suggested that the introduced *hGM-CSF* was successfully transcribed in the transgenic plants. Thereafter, the transgenic plant, O64-8, was selected for the following experiments on the basis it had the highest level of the *hGM-CSF* transcript among the transformants.

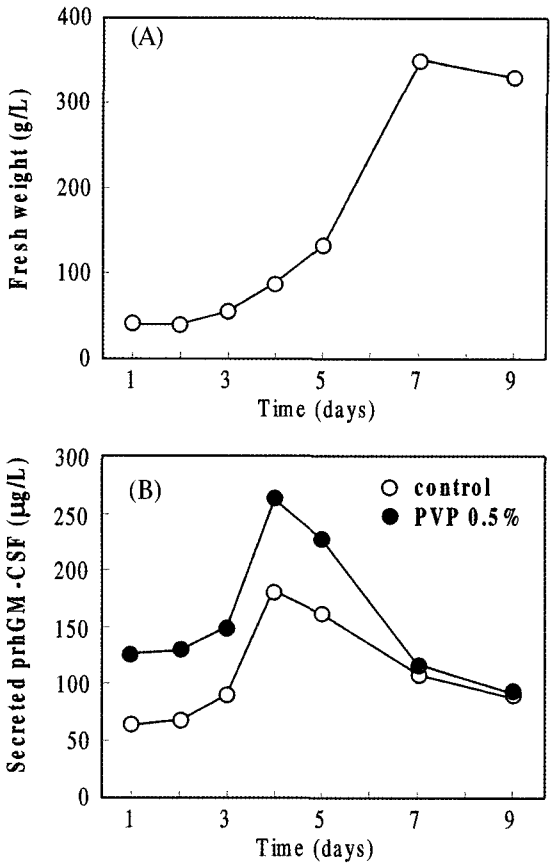

Fig. 3. The effects of the addition of PVP on the production of the prhGM-CSF in the plant cell suspension culture. The amount of prhGM-CSF was determined by ELISA as described in the Materials and Methods, and the time course of the prhGM-CSF production is indicated as a reference.

Quantitative and Western Blot Analyses of prhGM-CSF

To confirm the presence of the hGM-CSF produced by the transgenic tobacco cell suspension culture was analyzed by Western blot analysis and ELISA. The transgenic suspension cells derived from the O64-8 transgenic plant produced about 180 μg/L of prhGM-CSF 4 days after the initiation of the cell suspension culture (Fig. 3A and Fig. 4). After 5 days, however, the production of the prhGM-CSF produced from the transgenic tobacco cell suspension culture decreased as

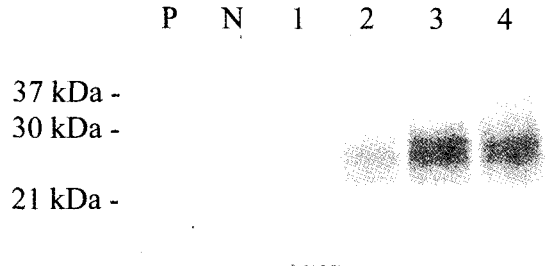

Fig. 4. Western blot analysis of the prhGM-CSF. Lanes P and N denote the positive control derived from *E. coli* and the non-transformants, respectively. Lanes 1, 2, 3 and 4 contain the total secreted protein from 2, 3, 4 and 5 day cultures of the transformant O64-8, respectively.

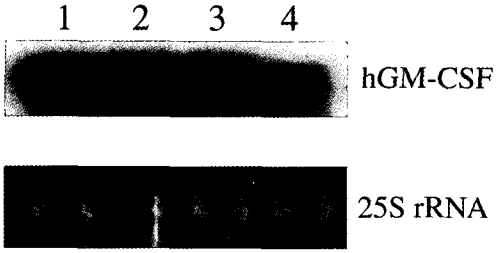

Fig. 5. Northern blot analysis to determine the temporal expression pattern of *hGM-CSF* during the plant cell suspension culture. Lanes 1, 2, 3 and 4 contain 15 μg total RNA extracted from 3, 5, 7 and 9 day cultures of the transformant O64-8, respectively. Loading standards were indicated by the ethidium bromide stained-rRNA in lower panel.

the cultivation time proceeded, while the growth of the transgenic suspension cell increased dramatically (Fig. 3B). However, the transcript levels of the *hGM-CSF* of transgenic suspension cells were still high at those times (Fig. 5). These results suggested the decreased production of prhGM-CSF might not represent the decreased prhGM-CSF gene expression. Rather, the produced prhGM-CSF might be unstable in the cell culture medium and the decreased levels of prhGM-CSF might be due to the decay of prhGM-CSF present in the culture supernatant. The *E. coli*-derived rhGM-CSF is 14 kDa without glycosylation but the molecular weight of natural hGM-CSF may be 24 kDa without or higher with oligosaccharide addition at two *N*-linked and five *O*-linked sites [1]. As shown in Fig. 4, the prhGM-CSF derived-from the plant cell suspension culture appeared as multiple bands between about 25 and 33 kDa. This result suggested that, the 25 to 33 kDa form represents correctly processed hGM-CSF, possibly differing by glycosylation level.

Effect of Protein Stabilizer on the prhGM-CSF Production

In order to confirm the above assumption, we tested if the addition of a protein stabilizer could increase the

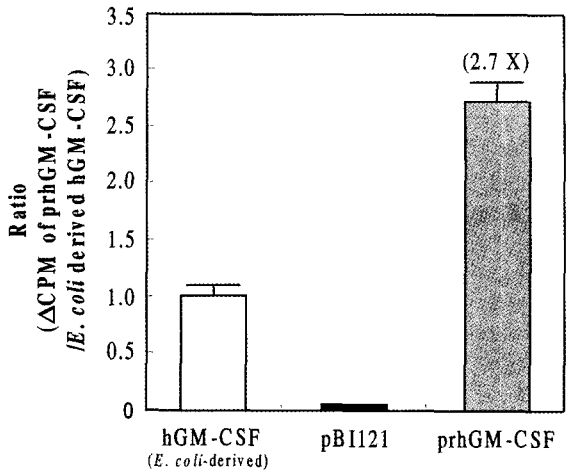

Fig. 6. Analysis of specific activity of prhGM-CSF produced from transgenic plant suspension cells. The 5 ng/mL of prhGM-CSF produced from the O64-8 cell line and *E. coli*-drieved hGM-CSF were used for the biological activity analysis. Culture medium of tobacco cells transformed with only the vector and commercial hGM-CSF were used as the negative control and the positive control, respectively. The data were obtained from the mean values of triplicate assays, and the error bars represent the standard error among the three separate experiments.

extracellular level of prhGM-CSF. To perform the experiment, polyvinylpyrolidine (PVP), as a protein stabilizer, was added in the culture medium at the beginning of the plant cell suspension cultures. As shown in the Fig. 4, the extracellular prhGM-CSF concentration was increased with the addition of PVP. The optimum PVP concentration was 0.5 g/L at which the amount of extracellular prhGM-CSF was increased up to about 270 μg/L (1.5-fold increased to the normal culture medium). Although the addition of PVP improved the prhGM-CSF production, the cell growth was not significantly affected (data not shown). These results suggest that the relatively low level of prhGM-CSF protein in the culture supernatant in compared to the level of *hGM-CSF* transcripts was due to the instability of produced prhGM-CSF.

Determination of the Biological Activity of prhGM-CSF

Biological activity of prhGM-CSF produced from plant cell suspension culture of transgenic tobacco was estimated by measuring the capability of the protein to support the growth of hGM-CSF dependent TF-1 cells. As shown in the Fig. 6, pBI121 transformed-samples, negative control, did not support the growth of hGM-CSF dependent TF-1 cells. However, the positive control rhGM-CSF (commercially-purchased recombinant hGM-CSF from *E. coli*) as well as the prhGM-CSF produced from the plant cell suspension culture of the transgenic tobacco efficiently supported the proliferation of the TF-1 cells. Interestingly, the specific activity of the prhGM-CSF from transgenic tobacco cell suspension culture,

estimated by the degree of [*methyl*-^{3}H] Thymi-dine uptake per ng of prhGM-CSF, was 2.7 times higher than that of the commercial GM-CSF. This result suggested that the specific activity of the prhGM-CSF produced through the plant cell suspension culture was higher than that produced through the *E. coli* expression system.

DISCUSSION

The plant cell suspension culture system for the production of useful foreign proteins has a significant advantage over those of whole plant biomass or whole cell lysates in the area of downstream purification. Recently, suspended plant cells have been used in several studies as a mean for producing a variety of foreign proteins. These included the recombinant antibodies [23-25], enzymes such as β-glucuronidase [26] and invertase [27], and proteins of therapeutic value such as human interleukin (IL)-2 and IL-4 [28], ricin [29], GM-CSF [6,7], and human α-antitrypsin [30]. However, a summary of these previous results has shown that the levels of the recombinant protein were significantly lower (approximately 180 to 300 μg/L) in plant cell suspensions than in whole plants or organ cultures such as leaves, seeds and hairy roots. Previous studies have shown that the productivity and stability of the recombinant proteins from the plant cell cultures were affected by proteases following secretion. In this study, the production of the prhGM-CSF from the transgenic suspension cells decreased as the cultivation time proceeded, while the transcript levels of the *h-GM-CSF* remained high during the same period. This suggests that the contact of the prhGM-CSF with proteases for long periods in the plant cell culture medium caused its degradation. Therefore, a continuous-type bioreactor might be effective in preventing the protease degradation of the secreted proteins. Otherwise, it might be effective to apply two strategies that Terashima *et al.* [31] used two strategies to produce the high level (85 mg/L) of AAT. For example, they used RAmy3D promoter, which is strongly and rapidly expressed promoter under the sugar starvation condition. Also, they induced the expression of the AAT gene after the lag phase of cell growth for only 48 hrs to reduce the time for the contact between the produced AAT and proteases.

In the studies for enhanced recovery of secreted proteins into the plant cell culture medium, it has been reported that the use of protein stabilizing agents such as PVP [23,32], polyethylene glycol [33-35] gelatin [23], and bacitracin [36,37] could enhance the stability of the produced proteins. Magnuson *et al.* [32] reported that the use of PVP as a protein stabilizing agent improved antibody titres in the culture fluid by up to 35-fold. The results presented here demonstrate that with the addition of 0.5 g/L protein stabilizing agent, PVP, to the liquid medium, a slightly enhanced recovery (about 1.5-fold) of the secreted prhGM-CSF was attained without adversely affecting the growth of the transgenic cells. These results also suggest that the protein stabilizing agent used in this study might not be a good agent to prevent the prhGM-CSF from denaturation. In other studies, the degradation of human AAT in the medium of transgenic rice cell cultures was attributed to the proteases released from disrupted cells. Hence, adjusting the medium osmotic pressure inhibited cell disruption and improved the active protein titre [31]. In this regard, further works are required to enhance the ability to produce high levels of proteins and to increase the stability of secreted proteins in the liquid medium.

The specific activity of the prhGM-CSF produced by the plant cell culture was 2.7 times higher than that of a commercially available rhGM-CSF (Fig. 6), which suggested the prhGM-CSF produced by the plant cell culture may have favorable characteristics over that of the *E. coli*-derived rhGM-CSF. It has been reported that the specific activity of the murine GM-CSF from a recombinant *A. niger* strain was two and a half times greater than that of commercially available GM-CSF [5]. As nonglycosylated *E. coli*-derived, and the differently glycosylated yeast-, *A. niger*- and the plant-derived, recombinant GM-CSF are all biologically active, the *N*-linked glycosylation does not appear to be essential for GM-CSF activity. Therefore, further biochemical characterization of the secreted prhGM-CSF is still required to determine the extent of posttranslational modification (*i.e.*, glycosylation) performed by the plant cells. Cytokines are known to have many pleotrophic effects on immuno responses, however, the clinical applications of many recombinant therapeutic human cytokines have been hampered by their side effects due to overdose [38-40]. Therefore, the increase in the specific activity is important for the clinical application of many recombinant cytokines.

The present results have clearly shown that the prhGM-CSF, with a high specific biological activity from plant cells can be successfully produced and derived for secretion into the culture medium, and its specific activity is greater than that derived from *E. coli*.

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