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Production of Useful Proteins by Plant Cell Culture

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Abstract

Plant cell culture is emerging to express bioactive foreign proteins because it has several advantages in that it is safe, economical, genetically stable and eukaryotic expression system comparing with other expression systems. However several limitations such as slow growth rate, low expression level and lack of well established down stream process need to be answered. As a preliminary approach to produce the immunologically interested molecules through the plant cell culture, we tested if granulocyte-macrophage colony stimulating factors (GM-CSFs) from both murine (mGM-CSF) and human (hGM-CSF) are produced as a biologically active form through plant cell culture. The murine and human GM-CSF genes were cloned into the plant expression vector, pBI121, and Ti-plasmid mediated transformation of tobacco leaves was conducted using *Agrobacterium tumefaciens* harboring both recombinant GM-CSF (rGM-CSF) genes. Cell suspension culture was established from the leaf-derived calli of transgenic tobacco plant. Northern blot analysis indicated the expression of the introduced mGM-CSF gene in both transgenic plant and cell suspension cultures. In addition, the biological activities of both murine and human GM-CSF from plant cell culture were confirmed by measuring the proliferation of the GM-CSF dependent FDC-P1 and TF-1 cells, respectively.

Abbreviations: crmGM-CSF, recombinant human granulocyte-macrophage colony stimulating factor purchased from commercial source; mGM-CSF, murine granulocyte-macrophage colony stimulating factor; hGM-CSF, human granulocyte-macrophage colony stimulating factor; prmGM-CSF, recombinant mouse granulocyte-macrophage colony stimulating factor produced by plant cell suspension culture; prhGM-CSF, recombinant human granulocyte-macrophage colony stimulating factor produced by plant cell suspension culture; rmGM-CSF, recombinant murine granulocyte-macrophage colony stimulating factor; rhGM-CSF, recombinant human granulocyte-macrophage colony stimulating factor; 3H-TdR, [methyl-3H]Thymidine deoxyribose

A variety of bioactive compounds have been stably expressed in plants using molecular biological techniques [19, 21, 7, 23]. Production of valuable proteins through the plant cell culture has several advantages over either prokaryotic or animal cell expression system [9]. They are generally inexpensive to grow on a large scale, and their production is not limited to fermenter capabilities since they have high cell density [11]. In addition, since the plant cell culture is an eukaryotic expression system, post-translational modification in plant cell culture system is more similar than those of prokaryotic or other eukaryotic expression system, which allows it to produce more complicated target proteins as biologically active ones. It is possible to integrate many different target proteins into a single cell line through a conventional plant breeding techniques. Also, the proteins produced through plant cell culture is safer than those through prokaryotic and animal expression system, especially in the proteins applied for human disease treatment. In addition, plant cell culture system is more affordable to human society than those of transgenic plant because it is possible to direct the target compound to culture medium, which allows us to purify the compound instead of using whole engineered plant. Finally, purification procedure of the produced proteins through plant cell culture is easy and economical, since there are less unwanted protein components in the culture media than those in prokaryotic and animal cell expression system.

However there are several limitations comparing with other expression system in that plant cells have slow growth rate than the other expression system, expression level of foreign protein using plant cell culture is quite low and a general procedure for large scale production is not well established.

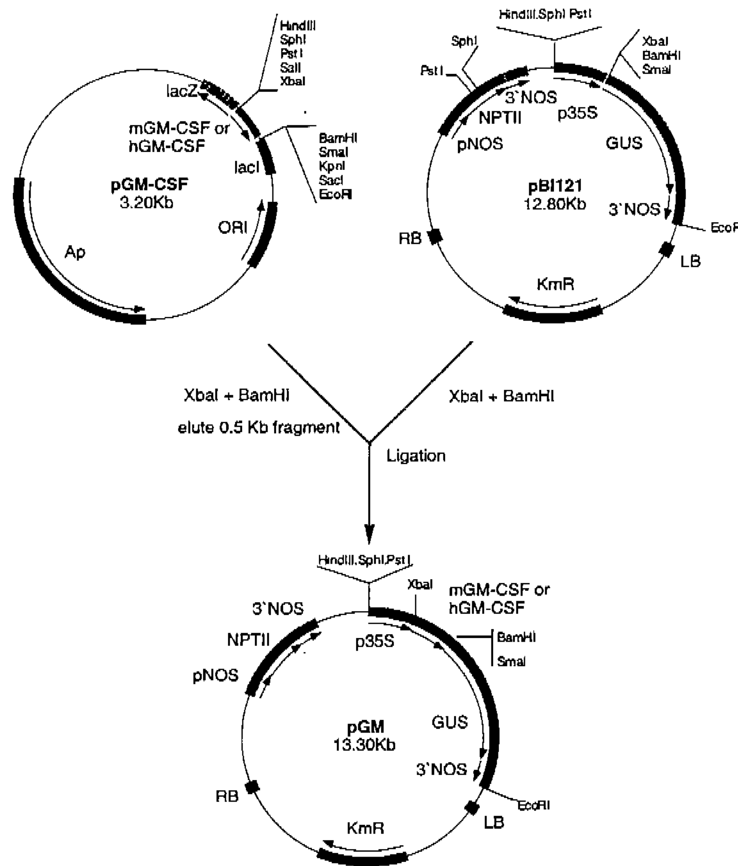

Figure 1. Cloning strategy to generate the plant expression vector, pGM, containing mGM-CSF or hGM-CSF cDNA. Cloned mGM-CSF and hGM-CSF genes are transcriptionally fused to β -glucuronidase gene and placed under the control of cauliflower mosaic virus 35S promoter Arrows indicate the direction of transcription and closed bars represent the coding genes.

In this review, I will explain the prospects of production of useful protein using plant cell culture with the example of granulocyte-macrophage colony stimulating factor (GM-CSF) production through tobacco cell culture.

As an initial attempt to produce valuable immunological molecules, such as antibodies and cytokines, we tried to produce both murine GM-CSF (mGM-CSF) and human GM-CSF (hGM-CSF), which do not exhibit cross-activity, from plant cell suspension culture. By transferring the GM-CSF gene to tobacco cells, expression of the transferred GM-CSF gene was confirmed by Northern blot analysis in mRNA level. Moreover, produced rGM-CSF was biologically fully active in that it sustained the proliferation of GM-CSF dependent FDC-P1 cells.

Granulocyte-macrophage colony stimulating factor (GM-CSF) is one of the first of large number of cytokines purified and cloned [18]. The structures of GM-CSFs from several organisms including mouse have been determined by deduction of the amino acid sequence from the nucleotide sequence of cDNA clones [8, 20, 13, 22, 14]. The mature mGM-CSF comprises 124 amino acid residues while hGM-CSF consists of 127 residues, which are preceded by a hydrophobic leader sequence of 25 amino acid residues in length. The murine and human GM-CSF polypeptide sequences both contain two potential N-linked glycosylation sites and the isolated GM-CSF from both source is glycosylated to approximately 23 kDa [16]. GM-CSF can be produced by a number of different cell types, including T lymphocytes, macrophages, endothelial cells and fibroblasts, under different circumstances. The major actions of GM-CSF involve the regulation of survival, differentiation, proliferative and functional activities in granulocyte-macrophage populations. Hence, there are at least three major areas in which GM-CSF has been considered for clinical use: amelioration of acute and chronic states of neutropenia, including facilitation of

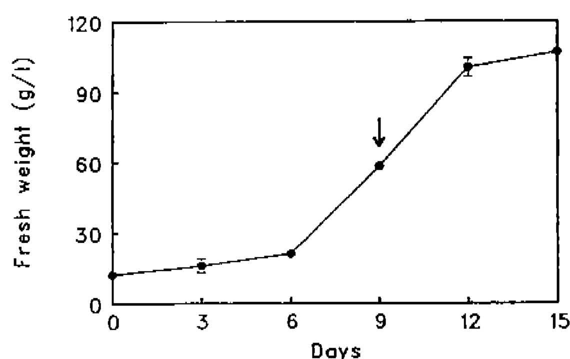

Figure 2. Growth curve of plant cell suspension culture. Transgenic suspension cell lines were obtained from the leaf-derived calli of transgenic plants and suspension cell culture was established in MS medium containing 2 mg/l of 2,4-dichlorophenoxyacetic acid, 0.02 mg/l of kinetin and 3 % sucrose. The suspension cell culture was kept by transferring a fifth of proceeding culture onto fresh medium every 12 days. Plant cell suspension culture for the experiments was started at 50 ml scale with 12 g/l wet weight of cultured cell as an inoculum size. The arrow represents the time point for sample preparation for further experiments. The data represent the mean values of the triplicates.

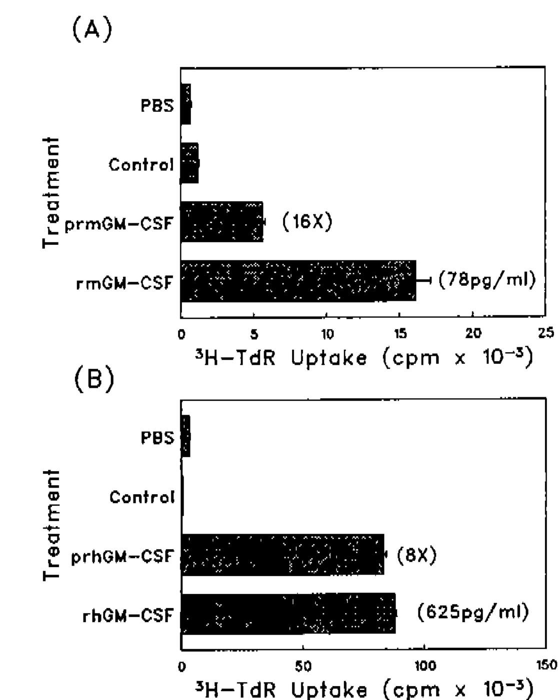

Figure 3. Biological activity of the recombinant GM-CSFs produced by plant cell suspension culture. (A) Biological activity of the recombinant mouse GM-CSF (prmGM-CSF) has been assayed by measuring the ability of recombinant proteins to support the proliferation of mGM-CSF dependent FDC-P1 cells (Metcalf, 1989). Briefly, 5 × 10^3 FDC-P1 cells were culture for 24 h in the presence of each samples and then 1 μCi of [methyl-^{3}H]Thymidine was added and incubated for another 12 h. After harvesting the cells, the level of tritium uptake has been measured by using liquid scintillation counter to determine the level of proliferation. PBS, control, prmGM-CSF and rmGM-CSF represent the PBS negative control for dependent cell proliferation assay, the sample from mock-transformed plant cell suspension culture, 16X diluted sample of recombinant mouse GM-CSF produced by plant cell suspension culture and 78pg/ml of commercially purchased mouse GM-CSF which has been produced by using *E. coli* expression system, respectively. (B) Biological activity of the recombinant human GM-CSF (prhGM-CSF) has been assayed similarly as prmGM-CSF except using TF-1 cells instead FDC-P1 as human GM-CSF dependent cells (Kitamura *et al.*, 1989; Drexler *et al.*, 1997). PBS, control, phmGM-CSF and hmGM-CSF represent the PBS negative control for dependent cell proliferation assay, the sample from mock-transformed plant cell suspension culture, 8X diluted sample of recombinant human GM-CSF produced by plant cell suspension culture and 625pg/ml of commercially purchased mouse GM-CSF which has been produced by using *E. coli* expression system, respectively.

bone marrow and peripheral blood stem cell transplantation; adjunctive therapy with anti-microbials in nonneutropenic states; and anti-neoplastic effects via differentiation of the malignant phenotype, recruitment of leukaemic cells to enhance the efficacy of chemotherapy or enhancement of anti-tumor activity [18]. In addition, anti-tumor activity of GM-CSF against several tumor types including solid tumors has been reported recently [5, 1, 17, 4]. From early 1993 in the USA, recombinant human GM-CSF has been approved for use as a means of accelerating engraftment following autologous bone marrow transplantation in patients with lymphoid malignancies.

Vector construction and establishment of suspension cell culture

The 500 bp *XbaI/Bam*HI DNA fragments containing either mGM-CSF or hGM-CSF cDNA was cloned into the corresponding cloning site of pBI121 vector [10, 3] to generate pGM. GM-CSF gene is placed under the control of cauliflower mosaic virus 35S promoter and fused transcriptionally to β-glucuronidase (GUS) gene (Figure 1). *Agrobacterium*-mediated transformation with pGM was applied [2]. Suspension cell lines were obtained from the leaf-derived calli of plant and suspension cell culture was established in MS medium containing 2 mg/l of 2,4-dichlorophenoxyacetic acid, 0.02 mg/l of kinetin, and 3% sucrose. The suspension cell culture was kept alive by transferring a fifth of a proceeding culture onto fresh medium every 12 days and the growth rate of suspension cell culture was determined by measuring the fresh weight of cultured cells (Figure 2). In order for GM-CSF production occur in suspension culture, 12 g/l fresh weight of cultured cell was inoculated into a 250 ml flask containing 50 ml of MS medium. The sample was collected on the 9th day

Table 1. Quantitative analysis of expressed rGM-CSF

rGM-CSF	Cell type	
	Parental cell (μg/l)	Adaptaed cell (μg/l)
mGM-CSF	0.02	0.434
hGM-CSF	4.73	N.D.

N.D. indicates "not determined" because the improvement of productivity is undertaking.

after inoculation when cells were believed to be in the half-maximal growth stage (Figure 2).

Biological activity of rGM-CSF produced by plant suspension cell culture

In order to test if the recombinant GM-CSF (rGM-CSF) produced from plant cell suspension culture (prGM-CSF) is biologically active, the biological activities of murine and human GM-CSF were tested by measuring the capability of the protein to support the growth of GM-CSF dependent FDC-P1 and TF-1 cells, respectively (Figure 3). As shown in the Figure 3, all the negative controls, such as PBS and vector transformed-samples did not support the growth of GM-CSF dependent cells through out all the dilutions. However, the positive controls, commercially purchased rGM-CSFs (rmGM-CSF and rhGM-CSF), supported a significant level of GM-CSF dependent cell proliferation when it was treated with the rGM-CSF at the concentration of 0.5 μg/ml. Interestingly, samples prepared from the culture supernatant as well as the cell lysate of pGM transformed transgenic plant efficiently support the proliferation of GM-CSF dependent cells. These results show that both murine and human GM-CSF genes are expressed and stayed as a biologically active form during the plant cell suspension culture.

Quantitative analysis for the amount of expressed rGM-CSF protein produced from plant cell culture could be estimated by ELISA (Table 1). The initial amount of mGM-CSF was estimated to be 0.02 μg/l. However the yield of mGM-CSF was significantly increased due to the repeated selection of better adapted cell line. The recent productivity of mGM-CSF appears to be 0.43 μg/l. In addition, the initial amount of hGM-CSF was 4.73 μg/l, which is a lot higher than that of mGM-CSF. Improvement of hGM-CSF productivity is also currently undertaking.

Future studies

In order to enhance the productivity of target protein, several approaches are currently undertaken. First, many plants are under investigation to obtain the cell line with fast specific growth rate in the suspension culture. Several candidates with shortened cell cycle have been reported and those are wild type cultivars of tobacco plant and several different species of *Lemna* spp. including duckweed. These plants are currently suspension-cultured and maintained for several generations to compare the growth rate. Secondly, Expression level of foreign genes are improved by using strong promoters as well as enhancer elements. Expressed genes are screened by using expression sequence tag (EST) as well as RNA differential display in order to obtain the strong promoter specific to suspension culture. Several novel genes were identified from tobacco cell culture and are currently investigating to see whether they have better capability to drive the foreign gene expression than that of 35S promoter from Cauliflower Mosaic Virus. Enhancer elements of known promoters are under evaluation to see enhancing activity for foreign gene expression. Lastly, tissue culture techniques are under investigation to establish better adapted-cell line showing uniformed cell size and prolonged viability during cell culture. Mechanical sieving during successive transfer on the fresh medium and repeated plating on the MS salt media were able to establish a better adapted cell line which gives the 20 fold increase of GM-CSF productivity compared with the parental cell line (Table 1). In addition, stabilizers preventing the expressed product from degrading proteolytically and losing its three dimensional structure are also screening and optimization of culture medium is currently carrying out.

References

1. Abe J, Wakimoto H, Yoshida Y, Aoyagi M, Hirakawa K, Hamada H: J Cancer Res Clin Oncol 121: 587-592 (1995).
2. An G, Watson BD, Chianh CC: Plant Physiol 81: 301-305 (1988).
3. Datla RS, Hammerlindl JK, Panchuk LE, Keller W: Gene 122: 383-384 (1992).
4. Dong QG, Graziani A, Garlanda C, De Calmanovici RW, Arese M, Soldi R, Vecchi A, Mantovani A, Bussolino F: Int J Cancer 65: 700-708 (1996).
5. Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K, Jackson V, Hamada H, Pardoll D, Mulligan RC: Proc Natl Acad Sci USA 90: 3539-3543 (1993).

6. Drexler HG, Zaborski M, Quentmeier H: Leukemia 11: 701-708 (1997).
7. Fiedler U, Conrad U: Bio/Technology 13: 1090-1093 (1995).
8. Gough NM, Gough J, Metcalf D, Kelson A, Grail D, Nicola NA, Burgess AW, Dunn AR: Nature 309: 763-767 (1984).
9. Hughes J, Qoronfleh W: BioPharm 4: 18-26 (1991).
10. Jefferson RA, Kavanagh TA, Bevan MW: EMBO J 6: 3901-3907 (1987).
11. Krebbers E, Bosch D, Vandekerckhove J: in Palnt Protein Engineering (Shewry PR and Gutteridge S, eds) pp. 315-325, University Press, Cambridge (1992).
12. Kitamura T, Tojo A, Kuwaki T, Chiba S, Miyazono K, Urabe A, Takaku F: Blood 73: 375-380 (1989).
13. Lee F, Yokota T, Otsuka T, Gemmell L, Larson N, Luh J, Arai K, Rennick D: Proc Natl Acad Sci USA 82: 4360-4364 (1985).
14. Maliszewski CR, Schoenborn MA, Cerretti DP, Wignall JM, Picha KS, Cosman D, Tushinski RJ, Gillis S, Baker PE: Mol Immunol 25: 843-850 (1988).
15. Metcalf D: Cancer Res 49: 2305-2311 (1989).
16. Nicola NA, Burgess AW, Metcalf D: J Biol Chem 254: 5290-5299 (1979).
17. Ragnhammar P: Med Oncol 13: 167-176 (1996).
18. Rasko JEJ, Gough NM: in The Cytokine Handbook (Thomson A, ed) pp. 343-369, Academic Press, London (1994).
19. Sijmons PC, Dekker BMM, Schrammeijer B, Verwoerd TC, van den Elzen PJM, Hoekema A: Bio/Technology 8: 217-221 (1990).
20. Sparrow LG, Metcalf D, Hunkapiller MW, Hool LE, Burgess AW: Proc Natl Acad Sci USA 82: 292-296 (1985).
21. Vandekerckhove J, Van Damme J, Van Lijsebettens M, Botterman J, De Block M, Vandewiele M, De Clercq A, Leemans J, Van Montagu M, Krebbers E: Bio/Technology 7: 929-932 (1989).
22. Wong GG, Witek JS, Temple PA, Wilkens KM, Leary AC, Luxenberg DP, Jones SS, Brown EL, Kay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM, Wang EA, Clark SC: Science 228: 810-815 (1985).
23. Wongsamuth R, Doran PM: Biotechnol Bioeng 38: 767-780 (1997).