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NOTE

A Recombinant Mouse GM-CSF Protein Expressed as an Inclusion Form Shows Colony Stimulating Activity

Jin-Kyoo Kim[1], Eun-Jung Sohn[1], Soo-O Lee[1], Choon-Taek Lee[2], Ah Young Lee, Hye Kyung Chung, Bong Whan Sung, and Hyun Joo Youn*

Department of Microbiology, College of Natural Sciences, Inje University, Kimhae 621-749, Korea
[1]Department of Microbiology, College of Natural Sciences, Changwon National University, Changwon 241-773, Korea
[2]Department of Internal Medicine, College of Medicine, Seoul National University, Seoul 110-744, Korea

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic hematopoietic growth factor and an activator of mature myeloid cells, and recombinant GM-CSF is increasingly under clinical studies for the treatment of various diseases including cancer, infectious diseases and hematopoietic diseases. We constructed a recombinant mouse GM-CSF expression plasmid with *pelB* leader sequence and His.Tag under T7 promoter control, and showed that the construct produced a 20 kDa recombinant protein in 8M urea. We also showed that the 20 kDa recombinant protein prepared in 8M urea stimulated colony formation *in vitro*, indicating that the recombinant mGM-CSF can be renatured to its native form to show the colony stimulating activity.

Key words: GM-CSF, recombinant protein, expression construct, His-Tag, colony stimulating activity

Colony-stimulating factors (CSFs) are cytokines which stimulate the expansion and differentiation of bone marrow progenitor cells. Different CSFs act on bone marrow cells at different developmental stages and promote specific colony formation of different lineages. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic hematopoietic growth factor and an activator of mature myeloid cells (3). It is produced by activated T cells, macrophages, endothelial cells and stromal fibroblasts, and acts on bone marrow cells to increase inflammatory leukocyte populations including granulocytes and macrophages (22, 27). GM-CSF also shows macrophage-activating factor activity *in vitro*, stimulating a variety of antimicrobial activities and potentiating inflammatory responses (4, 13, 16, 24, 25, 29). It is also thought to promote differentiation of Langerhans cells into dendritic cells and to recruit dendritic cells into tumor cells (21).

GM-CSF along with other cytokines is used to speed up bone marrow recovery after cancer chemotherapy and to harvest bone marrow stem cells in bone marrow transplantation (1, 17). It also has potential clinical significance in the management of inflammatory diseases and certain leukemias where GM-CSF plays a pathogenic role (8, 9, 10). Recent studies of GM-CSF secreting tumor cell vaccination and GM-CSF gene therapy showed potent anti-tumor immunity (11, 23, 26, 28), suggesting the potential use of GM-CSF in cancer treatment. Recently, recombinant GM-CSF is increasingly under clinical study for the treatment of various diseases including cancer, infectious diseases and hematopoeitic diseases, and becomes one of the most clinically successful application of any biological therapeutic agents (7, 12, 14, 19, 20). It also showed potential as a prospective adjuvant for vaccination (2, 5, 15).

The mouse GM-CSF (mGM-CSF) cDNA gene of pICmuGM-CSF plasmid was amplified using a set of PCR primers 5'-atcaccgccGCACCCACCCGCTCACCC-3' with a *NcoI* restriction site and 5'-atcaggtgaccTTTTGGACTGGTTTTTTGCATTC-3' with a *BstEII* restriction site. PCR amplifaication of pICmuGM-CSF plasmid DNA produced a single amplified DNA fragment of 0.4 Kb as expected. The 0.4 kb DNA fragment was successfully cloned into the *NcoI* and *BstEII* cloning sites of the pFCH plasmid (18) to produce the pFCHmGM-CSF construct. The pFCHmGM-CSF construct contains a *pelB* leader sequence, mGM-CSF coding region and His.Tag under control of the *lacZ* promoter and *lac*I repressor. Expression of the recombinant mGM-CSF protein from the

* To whom correspondence should be addressed.
(Tel) 82-525-320-3265; (Fax) 82-551-279-7465
(E-mail) mbhjyoun@ijnc.inje.ac.kr

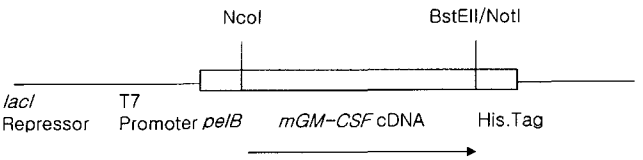

Fig 1. Schematic diagram of the linearized pETmGM-CSF expression construct. The mGM-CSF cDNA insert is cloned into the pET-22b(+) vector and is under the control of the T7 promoter and *lacI* repressor.

pFCHmGM-CSF construct was performed using the IPTG induction method as described by Kim *et al.* (18). 50 ml cultures were grown in the medium supplemented with glucose and ampicillin to an OD650 of approximately 1.5. The cultures were then washed with 25 ml of glucose free medium and the resulting cell pellets were resuspended in a fresh medium supplemented with IPTG and ampicillin to induce cloned mGM-CSF expression. After 5 and a half hours of induction, the cells were harvested and subjected to protein extraction from the periplasmic space, cytoplasm and cellular inclusion as described by Kim *et al.* (18). For the periplasmic protein extraction, cell pellets were subjected to osmotic shock treatment with TES buffer (0.5M sucrose, 0.1mM EDTA, 0.2M Tris-Cl, pH7.4) for 40 min. The remaining cell pellets were subjected to sonication protein preparation using sonication buffer (0.25M NaCl, 0.05M Tris, pH7.5), and the insoluble remnants were dissolved in urea denaturing buffer (8M urea, 0.1M NaH2PO4, 0.01M Tris pH8.0). The extracted protein preparations were then subjected to Ni-NTA agarose purification as described by the manufacturer (Qiagen, USA), and the recombinant protein expressions were examined. However, the production of recombinant mGM-CSF protein from the pFCHmGM-CSF expression construct appeared not to be successful. Ni-NTA purification of any of periplasmic, cytoplasmic and urea preparations did not yield a significant protein band of approximately 20 KDa, indicating failure of recombinant mGM-CSF expression using the lacZ promoter system (data not shown).

Since the production of the recombinant mGM-CSF expression was not obtained using the pFCHmGM-CSF construct, we prepared another expression construct under T7 promoter control. The mGM-CSF cDNA insert was cleaved out from the pFCHmGM-CSF construct using *NcoI* and *NotI* restriction enzymes and subcloned into the corresponding restriction sites of the pET22b(+) vector (Novagen, Germany), resulting in the pETmGM-CSF expression construct (Fig 1). As the pFCHmGM-CSF construct, the pETmGM-CSF construct contains a *pelB* leader and a His.Tag, while possessing a T7 promoter instead of a *lacZ* promoter under control of *lacI* repressor. The pETmGM-CSF construct was induced by IPTG and examined for recombinant mGM-CSF production as described above. After induction, protein extractions from

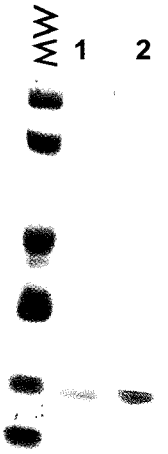

Fig 2. SDS-PAGE and Western blot analyses of the purified recombinant mGM-CSF protein from pETm GM-CSF. MW represents molecular weight standard of 94, 67, 43, 30, 20, and 14 KDa proteins. Lanes 1 and 2 represent SDS-PAGE of the Ni-NTA purified recombinant protein from the 8M urea preparation and Western blotting with His.Tag specific antibody, respectively. The image was processed using Gel-Doc System (Bio-Rad, USA).

periplasmic space, cytoplasm and urea treatment were prepared and subjected to Ni-NTA agarose purification. Although, significant amounts of 20 kDa recombinant proteins were not detected in periplasmic and cytoplasmic preparations, a single and strong protein band of 20 kDa was detected in the 8M urea preparation (Fig 2), indicating that recombinant mGM-CSF proteins may be expressed as an insoluble inclusion form. The 20 kDa protein in the urea preparation was further characterized using ELISA and Western blot assays using His.Tag and mGM-CSF specific antibodies. Western blot assay using His.Tag specific antibody (Sigma) revealed interaction of the 20 kDa protein band to the antibody, indicating that the 20 KDa protein possesses the His.Tag (Fig 2). ELISA assay using the mGM-CSF specific antibody (Endogen, USA) also showed significant reactivity of the protein to the antibody (Data not shown), indicating that the 20 kDa protein in the urea preparation could be the recombinant form of mGM-CSF. This study clearly showed recombinant mGM-CSF could be expressed using *pelB* leader and T7 promoter system, providing the possibility of the application of this system to other protein expression including recombinant human GM-CSF.

Colony stimulating activity of the purified 20 kDa recombinant mGM-CSF proteins from the urea preparation of the pETmGM-CSF construct was also examined. Since the recombinant mGM-CSF proteins in 8M urea were apparently denatured due to the high concentration of urea, we tried to renature the recombinant protein using dialysis in PBS. The 20 kDa recombinant protein was not precipitated but well solubilized in PBS and the colony stimulating activities of the PBS solublized protein prep-

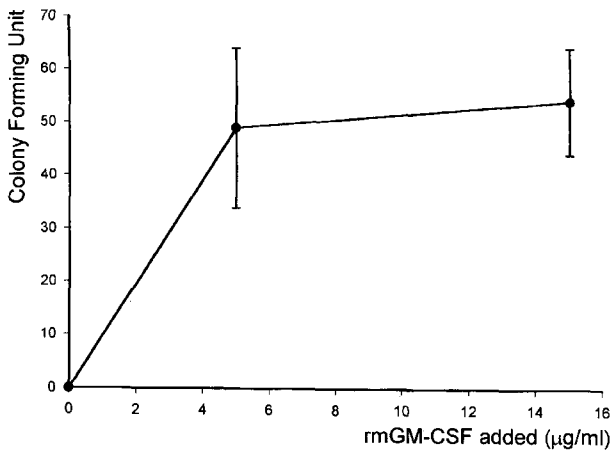

Fig 3. Colony stimulating activity of the purified 20 kDa recombinant mGM-CSF protein.

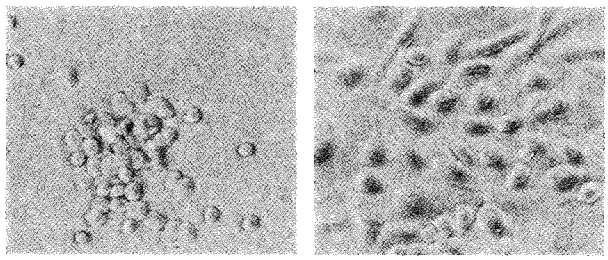

Fig 4. Cellular morphologies of the representative colonies from the recombinant mGM-CSF treated bone marrow cell culture. Generally, colonies with spherical cells (a) and mixed cells (b) were predominant in the assay.

arations were examined in triplicates in 24 well plates as described by Garland (29). Bone marrow cells from six week old BALB/c mouse were resuspended in complete DMEM (Sigma) medium supplemented with 10% FCS and the 1 ml of the bone marrow cell suspension was then plated into the wells (2×10^6 cells/well). To the wells, 0, 5 or 15 μg of dialyzed Ni-NTA purified proteins in PBS were added and then solidified with 2.2% low melting temperature agarose. The wells were incubated at 37°C in humidified atmosphere containing 5% CO_2. One week later, the numbers of colonies were recorded at the 5 random sites of each well and the average numbers of the resulting colonies were compared. The cellular morphologies of the representative colonies were also examined. Additions of purified 20 kDa protein at concentrations of 5 and 15 μg/ml significantly increased colony formation compared to that of the negative control (Fig 3 and 4), indicating that the 20 kDa protein from the urea preparation possessed colony stimulating activity. It also suggest that the denatured 20 kDa recombinant mGM-CSF protein in the 8M urea preparation was rightfully renatured to a biologically active form through simple dialysis.

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References

1. Aglietta, M., F. Montemurro, F. Fagioli, C. Volta, B. Botto, M. Cantonetti, V. Racanelli, L. Teofili, R. Ferrara, S. Amadori, G.L. Castoldi, F. Dammacco, and A. Levis. 2000. Short term treatment with Escheria coli recombinant human granulocyte-macrophage-colony stimulating factor prior to chemotherapy for Hodgkin disease. *Cancer* 88, 454-460.
2. Anandh, U., B. Bastani, and S. Ballal. 2000. Granulocyte-Macrophage Colony Stimulating Factor as an adjuvant to Hepatitis B vaccination in maintenance hemodialysis patients. *Am. J. Nephrol.* 20, 53-56.
3. Arai, K., F. Lee, A. Miyajima, S. Miyatake, N. Arai, and T Yokota. 1990. Cytokines: coordinators of immune and inflammatory response. *Ann. Rev. of Biochem.* 59, 783-836.
4. Bischof, R.J., D. Zafiropoulos, J.A. Hamilton, and I.K. Campbell. 2000. Exacerbation of acute inflammatory arthritis by the colony-stimulating factors CSF-1 and granulocyte macrophage (GM)-CSF: evidence of macrophage infiltration and local proliferation. *Clin. Exp. Immunol.* 119, 361-367.
5. Elsasser, D., H. Stadick, J.G. van de Winkel, and T. Valerius. 1999 GM-CSF as adjuvant for immunotherapy with bispecific antibodies. *Eur. J. Cancer* 35 Suppl. 3, S25-28.
6. Garland, J. 1987. Assays for interleukin3 and other myeloid colony-stimulating factors. p.303-322. *In* Lymphokines and Interferons: *A Practical Approach.* Clemens, Morris AG, and Gearing AJH (eds), IRL Press, Oxford
7. Groenewegen, G. and G.C. de Gast. 1999. GM-CSF can cause T cell activation; results of sequential chemo-immunotherapy. *Eur. J. Cancer* 35 Suppl. 3, S23-24.
8. Hercus, T.R., C.J. Bagley, B. Cambareri, M. Dottore, J.M. Woodcock, M.A. Vadas, M.F. Shannon, and A.F. Lopez. 1994. Specific human granulocyte-macrophage colony stimulating factor antagonists. *Proc. Natl. Acad. Sci. USA.* 91, 5838-5842.
9. Herranz, P., J.R. Arribas, A. Navarro, J.M. Pena, J. Gonzalez, F.A. Rubio, and M. Casado. 2000. Successful treatment of aphthous ulcerations in AIDS patients using topical granulocyte-macrophage colony-stimulating factor. *Br. J. Dermatol.* 142, 171-176.
10. Holland, S.M. 2000. Cytokine therapy of mycobacterial infections. *Adv. Intern. Med.* 45, 431-452.
11. Hurwitz, A.A., T.F.-Y. Yu, D.R. Leach, and J.P. Allison. 1998. CTLA-4 blockade synergizes with tumor-derived granulocyte-macrophage colony-stimulating factor for treatment of an experimental mammary carcinoma. *Proc. Natl. Acad. Sci. USA.* 95, 10067-10071.
12. Janik, J.E., L.L. Miller, W.C. Kopp, D.D. Taub, H. Dawson, D. Stevens, P. Kostboth, B.D. Curti, K.C. Conlon, B.K. Dunn, S.E. Donegan, R. Ullrich, W.G. Alvord, B.L. Gause, and D.L. Longo. 1999. Treatment with tumor necrosis factor-alpha and granulocyte-macrophage colony-stimulating factor increases epidermal Langerhans' cell numbers in cancer patients. *Clin. Immunol.* 93, 209-221.
13. Jarmin, D.I., R.J. Nibbs, T. Jamieson, J.S. de Bono, and G.J. Graham. 1999. Granulocyte macrophage colony-stimulating factor and interleukin-3 regulate chemokine and chemokine

receptor expression in bone marrow macrophages. *Exp. Hematol.* 27, 1735-1745.
14. Jones, T.C. 1999. Use of granulocyte-macrophage colony stimulating factor (GM-CSF) in prevention and treatment of fungal infections. *Eur. J. Cancer* 35 Suppl. 3, S8-10.
15. Kapoor, D., S.R. Aggarwal, N.P. Singh, V. Thakur, and S.K. Sarin. 1999. Granulocyte-macrophage colony stimulating factor enhances the efficacy of hepatitis B virus vaccine in previously unvaccinated haemodialysis patients. *J. Viral Hepat.* 6, 405-409.
16. Kedzierska, K., J. Mak, A. Mijch, I. Cooke, M. Rainbird, S. Roberts, G. Paukovics, D. Jolley, A. Lopez, and S.M. Crowe. 2000. Granulocyte-macrophage colony stimulating factor augments phagocytosis of *Mycobacterium avium* complex by human immunodeficiency virus type 1-infected monocytes/macrophages in vitro and in vivo. *J. Inf. Dis.* 181, 390-394.
17. Keller, J.R., S.E.W. Jacobsen, K.T. Sill, L.R. Ellingsworth, and F.W. Ruscetti. 1991. Stimulation of granulopoiesis by transforming growth factor β: Synergy with granulocyte/macrophage colony stimulating factor. *Proc. Natl. Acad. Sci.USA.* 88, 7190-7194.
18. Kim, J.K., M.F. Tsen, and E.S. Ward. 1994. Catabolism of the murine IgG1 molecule: evidence that both CH2-CH3 domain interfaces are required for persistence of IgG1 in the circulation of mice. *Scand. J. Immunol.* 40, 457-465.
19. Mangi, M.H. and A.C. Newland. 1999. Febrile neutropenia: prophylactic and therapeutic use of GM-CSF. *Eur. J. Cancer* 35 Suppl. 3, S4-7.
20. Mellstedt, H., J. Fagerberg, and A. Osterborg. 1999. Local low-dose of soluble GM-CSF significantly augments an immune response against tumour antigens in man. *Eur. J. Cancer* 35 Suppl. 3, S29-32.
21. Nasi, M.L., P. Lieberman, K.J. Busam, V. Prieto, K.S. Panageas, J.J. Lewis, A.N. Houghton, and P.B. Chapman. 1999. Intradermal injection of granulocyte-macrophage colony-stimulating factor (GM-CSF) in patients with metastatic melanoma recruits dendritic cells. *Cytokine Cell. Mol. Ther.* 5, 139-144.
22. Nichilo, M.O.D., and G.F. Burns. 1993. Granulocyte-macrophage and macrophage colony stimulating factors differentially regulates integrin expression on cultured human macrophages. *Proc. Natl. Acad. Sci. USA.* 90, 2517-2521.
23. Ozawa, H, W. Ding, H. Torii, J. Hosoi, K. Seiffert, K. Campton, N.R. Hackett, N. Topf, R.G. Crystal, and R.D. Granstein. 1999. Granulocyte-macrophage colony stimulating factor gene transfer to dendritic cells or epidermal cells augments their antigen-presenting function including induction of anti-tumor immunity. *J. Invest. Derm.* 113, 999-1005.
24. Sasaki, T., S. Horiuchi, M. Yamazaki, and S. Yui. 1999. Induction of GM-CSF production of macrophages by advanced glycation end products of the Maillard reaction. *Biosci. Biotechnol. Biochem.* 63, 2011-2013.
25. Schafer, K., S. Stoteknuel, P. Schollmeyer, and G.J. Dobos. 1998. Granulocyte macrophage-colony stimulating factor stimulates secretion of chemoattractive cytokines by peritoneal macrophages of CAPD patients. *Adv. Perit. Dial.* 14, 164-167.
26. Soiffer, R., T. Lynch, M. Mihm, K. Jung, C. Rhuda, J.C. Schmollinger, F.S. Hodi, L. Liebster, P. Lam, S. Mentzer, S. Singer, K.K. Tanabe, A.B. Cosimi, R. Duda, A. Sober, A. Bhan, J. Daley, D. Neuberg, G. Parry, J. Rokovich, L. Richards, J. Drayer, A. Berns, S. Clift, L.K. Cohen, R.C. Mulligan, and G. Dranoff. 1998. Vaccination with irradiated autologous melanoma cells engineered to secrete human granulocyte-macrophage colony-stimulating factor generates potent antitumor immunity in patients with metastatic melanoma. *Proc. Natl. Acad. Sci. USA.* 95, 13141-13146.
27. Staynov, D.Z., D.J. Cousins, and T.H. Lee. 1995. A regulatory element in the promoter of the human granulocyte-macrophage colony stimulating factor gene that has related sequences in other T-cell expressed cytokine genes. *Proc. Natl. Acad. Sci. USA.* 92, 3606-3610.
28. Suh, K.W., S. Piantadosi, H.A. Yazdi, D.M. Pardoll, H. Brem, and MA Choti. 1999. Treatment of liver metastases from colon carcinoma with autologous tumor vaccine expressing granulocyte-macrophage colony-stimulating factor. *J. Surg. Oncol.* 72, 218-224.
29. Williams, M.A., S.M. Kelsey, and A.C. Newland. 1999. GM-CSF and stimulation of monocyte/macrophage function in vivo relevance and in vitro observations. *Eur. J. Cancer* 35 Suppl. 3, S18-22.