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NOTE

A Recombinant Human GM-CSF Protein Expressed as an Inclusion form in *Escherichia coli* Stimulates Colony Formation and Cell Proliferation *in vitro*

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic hematopoietic growth factor involved in the development of myeloid cells from bone marrow, and an activator of mature myeloid cells functioning in a variety of antimicrobial and inflammatory responses. Recently, recombinant GM-CSF is increasingly under clinical study for treatment of various diseases including cancer, infectious diseases and hematopoietic diseases as well as for an immune response modulator. In this study, we constructed a recombinant human GM-CSF (rhGM-CSF) expression plasmid with a *pelB* leader sequence and His.Tag under T7 promoter control. The expression construct was shown to produce a recombinant protein of 20 kDa in the 8M urea preparation, indicating the rhGM-CSF may be expressed as an insoluble inclusion form. The 20 kDa recombinant protein in 8M urea was transformed into the water-soluble form by dialysis against PBS buffer (phosphate buffered saline). The soluble rhGM-CSF protein was shown to stimulate colony formation and cell proliferation *in vitro*, indicating that the rhGM-CSF could be refolded into its native form to show colony stimulating activity.

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

Colony-stimulating factors (CSFs) are cytokines stimulating 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 hematopoietic cell lineages. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic hematopoietic growth factor and an activator of mature myeloid cells (Arai *et al.*, 1990). 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 (Nichilo *et al.*, 1993; Staynov *et al.*, 1995). GM-CSF also shows macrophage-activating factor activity *in vitro*, stimulating a variety of antimicrobial activities and potentiating inflammatory responses (Schafer *et al.*, 1998; Jarmin *et al.*, 1999; Sasaki *et al.*, 1999; Williams *et al.*, 1999; Bischof *et al.*, 2000; Kedzierska *et al.*, 2000). It is also thought to promote differentiation of Langerhans cells into dendritic cells and to recruit dendritic cells into tumor cells (Nasi *et al.*, 1999).

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 (Keller *et al.*, 1991; Aglietta *et al.*, 2000). It also has potential clinical significance in the management of inflammatory diseases and certain leukemias where GM-CSF plays a pathogenic role (Hercus *et al.*, 1994; Herranz *et al.*, 2000; Holland, 2000). Recent studies of GM-CSF secreting tumor cell vaccination and GM-CSF gene therapy showed potent antitumor immunity (Hurwitz *et al.*, 1998; Soiffer *et al.*, 1998; Ozawa *et al.*, 1999; Suh

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et al., 1999), 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 hematopoietic diseases, and has become one of the most clinically successful applications of any biological therapeutic agent (Groenewegen and de Gast, 1999; Janik *et al.*, 1999; Jones, 1999; Mangi and Newland, 1999; Mellstedt *et al.*, 1999). It has also showed potential as a prospective adjuvant for vaccination (Elsasser *et al.*, 1999; Kapoor *et al.*, 1999; Anandh *et al.*, 2000). In this study, we constructed a recombinant human GM-CSF (rhGM-CSF) expression construct and successfully purified rhGM-CSF protein from *Escherichia coli* using 8M urea. The 20 kDa recombinant protein in the 8M urea preparation was then dialyzed against PBS buffer to obtain soluble rhGM-CSF protein. The soluble rhGM-CSF protein was shown to stimulate colony formation and cell proliferation *in vitro*, indicating that the rhGM-CSF can be refolded into its native form to show colony stimulating activity.

The human GM-CSF cDNA coding region was amplified using a set of PCR primers, 5'-atcaccatggccGCACCCGCCCGCTCGCCC-3' containing an *NcoI* restriction site and 5' coding sequences and 5'-atcaggtgaccTCCTGGACTGGCTCCCAGCAGTC-3' possessing a *BstEII* restriction site and 3' coding sequences. PCR amplification of the human GM-CSF cDNA (gift from Dr. David P. Carbone of the University of Texas, Southwestern Medical Center) produced a single amplified DNA fragment of 380 bp coding mature human GM-CSF protein of 127 amino acids except leader peptides of the protein. The 380 bp DNA fragment was successfully cloned into the *NcoI* and *BstEII* cloning sites of the pFCH plasmid (Kim *et al.*, 1994) to produce pFCHhGM-CSF constructs. The pFCHhGM-CSF constructs contained a *pelB* leader sequence, human GM-CSF coding region and His. Tag under the control of *lacZ* promoter and *lacI* repressor. Expressions of the rhGM-CSF protein from the pFCHhGM-CSF constructs were performed using the IPTG induction method as described by Kim *et al.* (1994). 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 the fresh medium supplemented with IPTG and ampicillin to induce cloned hGM-CSF expression. After five and a half hours of induction, the cells were harvested and subjected to protein extractions from the periplasmic space, cytoplasm and cellular inclusion. For the periplasmic protein extraction, cell pellets were subjected to osmotic shock treatments with TES buffer (0.5M sucrose, 0.1 mM EDTA, 0.2M Tris-Cl, pH7.4) for 40 min. The remaining cell pellets were subjected to sonication protein preparations using sonication buffer (0.25M NaCl, 0.05M Tris, pH7.5), and the insoluble remnants were dissolved in denaturing urea buffer (8M urea, 0.1M NaH_2PO_4, 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 by SDS-PAGE and western blotting. The production of rhGM-CSF proteins from the pFCHhGM-CSF expression constructs, however, appeared not to be successful since the Ni-NTA agarose purifications of any of the periplasmic, the cytoplasmic and the urea preparations did not yield a significant protein band of approximately 20 kDa. The expression of the rhGM-CSF protein was not successful using the *lacZ* promoter system as in the case of recombinant mouse GM-CSF expression (Kim *et al.*, 2000).

Since the productions of the rhGM-CSF protein from the pFCHhGM-CSF constructs were not accomplished, we prepared another expression construct under T7 promoter control. The human GM-CSF cDNA insert was cleaved out from a pFCHhGM-CSF construct using *NcoI* and *NotI* restriction enzymes and subcloned into the corresponding restriction sites of the pET22b(+) vector

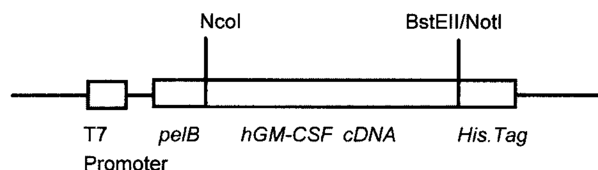

Fig 1. Schematic diagram of the linearized pEThGM-CSF expression construct. The human GM-CSF cDNA insert is cloned into the pET-22b(+) vector and is under the control of the T7 promoter and *lacI* repressor.

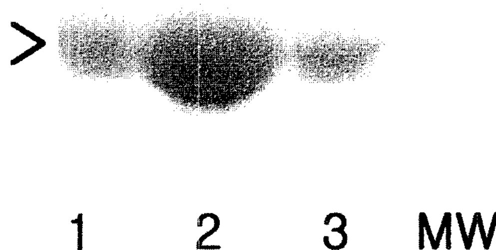

Fig. 2. SDS-PAGE analysis of the purified rhGM-CSF protein. MW represents molecular weight standard of 67, 43, 30, 20, and 14 kDa proteins. Lanes 1, 2 and 3 represent fractions of the Ni-NTA purified recombinant proteins from the 8M urea preparation. The image was processed using the Gel-Doc System (Bio-Rad, USA).

(Novagen, Germany), resulting in pEThGM-CSF expression constructs (Fig. 1). Like the pFCHhGM-CSF construct, the pEThGM-CSF construct contained a *pelB* leader and a His.Tag, while possessing a T7 promoter instead of a *lacZ* promoter under the control of *lacI* repressor. The pEThGM-CSF constructs were transformed into the *Escherichia coli* BL21(DE) (Novagen, Germany) and the transformants were induced by IPTG and examined for rhGM-CSF production as described above. After induction, protein extractions from the periplasmic space, cytoplasm and urea treatment were prepared and subjected to Ni-NTA agarose purifications. Although a significant amount of 20 kDa recombinant protein was not detected in the periplasmic and the cytoplasmic preparations, a strong protein band of 20 kDa was detected in the 8M urea preparation (Fig. 2), indicating that the 20 kDa rhGM-CSF protein could be expressed as an insoluble inclusion form. Western blot assay using His.Tag specific antibody (Sigma, USA) revealed interaction of the 20 kDa protein band to the antibody, indicating that the 20 kDa protein possessed the His.Tag (data not shown). This study showed that the rhGM-CSF protein could be expressed using the T7 promoter system instead of the *lacZ* promoter system, suggesting the expression of the rhGM-CSF protein might be influenced by the system employed.

Since it is assumed that the 20 kDa rhGM-CSF protein in 8M urea was apparently denatured due to the high concentration of urea, we tried to prepare soluble rhGM-CSF protein by renaturation of the 20 kDa protein using dialysis against PBS. Although some of the 20 kDa recombinant hGM-CSF protein was precipitated after dialysis, most of the protein was well solublized in PBS, providing renatured soluble 20 kDa rhGM-CSF protein as in the recombinant mouse GM-CSF preparation (Kim *et al.*, 2000). The colony stimulating activity of the soluble 20 kDa rhGM-CSF protein from the urea preparations was examined as described by Garland (1987) using human bone marrow cells and the human leukemic cell line HL60 (CCL-240, ATCC, USA). Human bone marrow cells from a leukemic patient were purified using Ficoll-Paque Plus (Pharmacia Biotech, Sweden) as described by the manufacturer. The purified bone marrow leukemic cells were resuspended in complete DMEM medium (Sigma) supplemented with 10% FCS and the cell suspensions were then plated into the cell wells (2×10^6 cells/ well). To each well, 8 or 16 μg of the soluble 20 kDa rhGM-CSF protein was added and the wells were then solidified with 2.2% of low melting temperature agarose. The HL60 cells were also resuspended in complete DMEM medium supplemented with 10% FCS and the cell suspensions were then plated into the cell wells (2×10^6 cells/well). To each well, 1.5, 3 or 6 μg of the soluble rhGM-CSF protein was added and then the wells were solidified as described above. The cell wells sup-

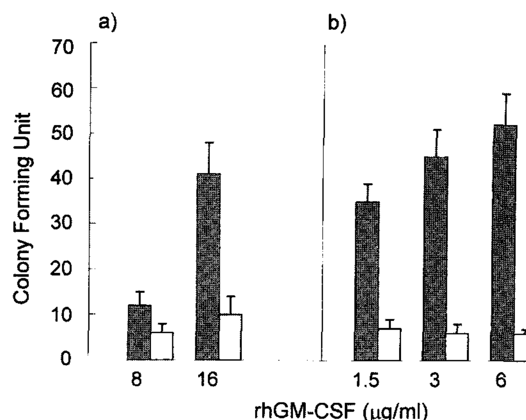

Fig. 3. Colony stimulating activity of the soluble rhGM-CSF protein. The colony stimulating activity of the soluble 20 kDa rhGM-CSF protein from the urea preparation was examined using human bone marrow cells (a) and human leukemic cell line HL60 (b). The black columns indicate the colony-forming units from the cultures supplemented with rhGM-CSF proteins while the white ones represent those from the control cultures supplemented with PBS.

plemented with PBS were used as negative controls and followed by the incubation at 37°C in a humidified atmosphere containing 5% CO_2. Five days later, the number of the colonies were scored at 5 random sites of each well and the average colony numbers were compared to the corresponding negative controls. Additions of 8 or 16 μg of soluble rhGM-CSF proteins to the human bone marrow cell cultures significantly increased colony formation compared to those of the negative control cultures (Fig. 3a). The cultures of HL60 supplemented with 1.5, 3 or 6 μg of the proteins also showed marked increases in the number of colonies compared to those of the control cultures (Fig. 3b), indicating that the soluble 20 kDa protein from the urea preparation possessed colony stimulating activity on human leukemic cells and the HL60 cell line.

The mitogenic activity of the soluble rhGM-CSF protein was also determined by cell proliferation assay using human leukemic cell line KG-1 (10246, KCLB, Korea) as described by Bradley (1980). Briefly, after incubation of KG-1 cells in 96-well microplates (10^5 cells/ well) for 48 hours in the presence or absence of 0.2, 0.4 or 0.8 μg of the rhGM-CSF protein, the wells were pulsed with 1μCi tritiated thymidine (^{3}H-TdR, Amersham, USA) for 16 hours without rhGM-CSF protein. After pulsing, the cells were harvested onto glass fiber filters (GF/A, Whatmann, USA) and counted for radioactivity associated with the cells. The 20 kDa rhGM-CSF protein promoted the growth of KG-1 cells at the concentrations employed in this study (Fig. 4), indicating the solubilized recombinant protein did retain its mitogenic activity on KG-1 cells.

In conclusion, we showed that the rhGM-CSF protein could be expressed in *Escherichia coli* using the T7 promoter system and urea extraction. The soluble rhGM-CSF

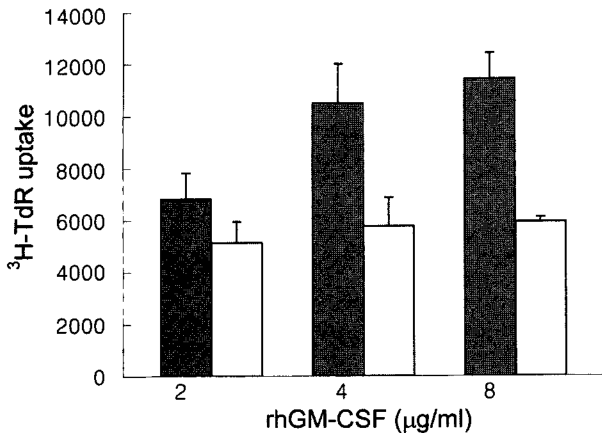

Fig. 4. The mitogenic activity of the soluble rhGM-CSF protein on human KG-1 cell line. To each cell well, 0.2, 0.4 or 0.8 μg of the soluble rhGM-CSF proteins was added and the wells supplemented with PBS were used as the controls. The black columns indicate amounts of tritiated thymidine incoorporation in the cultures supplemented with the soluble rhGM-CSF protein while the white ones represent those in the control cultures supplemented with PBS.

protein from the urea preparations also showed colony stimulating activity and mitogenic activity, indicating that the rhGM-CSF protein in 8M urea was correctly renatured to a biologically active form through simple dialysis.

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