Purification and Characterization of Recombinant Human Macrophase Colony-stimulating Factor Expressed in Chinese Hamster Ovary Cells

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We expressed human macrophase colony-stimulating factor (M-CSF) in Chinese hamster ovary (CHO) cells by introducing an expression plasmid coding for a 554-amino-acid M-CSF precursor and dihydrofolate reductase (DHFR) gene, and by amplifying the sequence. A cell line was obtained that secreted approximately 200,000 units/ml after 6 days in culture. The expressed recombinant human M-CSF (rhM-CSF) primarily consisted of two molecular species, a main 80–90-kD M-CSF as a homodimer and a molecular form higher than 150 kD. Purification of a main rhM-CSF gave an apparently homogeneous protein disulfide-bonded from 42-kD subunits, but one of the purified rhM-CSFs was composed of two subunit species with molecular masses of 44 and 42 kD. These purified rhM-CSFs had substantially the same specific activity (1 to 4 × 10^4 units/mg protein). Deglycosylation experiments with the latter rhM-CSF using chemical (trifluoromethanesulfonic acid) and enzymatic methods found a terminal neuraminic acid in addition to N- and O-glycosylation, but the two subunit species did not coalesce into a single molecule.

Macrophages are important in many aspects of the host defense immune response, and their production is regulated by a group of hematopoietic growth factors, called colony-stimulating factors (CSF). Among them, macrophase colony-stimulating factor (M-CSF), also called CSF-1, is essential role in the proliferation of precursor cells from bone marrow as well as in the differentiation of these precursor cells into mature macrophages. M-CSF also augments the effecter functions of blood monocytes and tissue macrophages, such as antifungal activity, antiviral activity, and antibody-independent and -dependent anti-tumor cytotoxicity. Furthermore, some reports suggest that M-CSF has other functions such as modulation of placental development, and regulation of blood cholesterol levels. Although some human cell lines produce M-CSF, the low level of production has hampered extensive in vivo studies of this factor. To further evaluate in vivo the roles of M-CSF, we sought to produce sufficient amounts of recombinant human M-CSF (rhM-CSF), which has physicochemical properties identical, or almost identical to those of native human M-CSF.

M-CSF is a heavily glycosylated, disulﬁde-bonded homodimeric protein. cDNAs for the M-CSF isolated from some M-CSF-producing cell lines have shown that, with regard to coding region, there are three types of mRNAs. These are produced by alternative splicing from a single gene, and encode 554-, 438-, and 256-amino-acid precursors, each of which is preceded by a 32-amino-acid signal sequence. Various host cells such as mammalian cells, insect cells, and E. coli cells have been used to express rhM-CSF. The most widely used mammalian expression system uses Chinese hamster ovary (CHO) cells as a host. Studies of the expression of M-CSF in CHO cells have, however, been reported only briefly. In this study, we expressed human M-CSF in CHO cells, and purified and characterized the recombinant proteins.

Materials and Methods

Construction of human M-CSF expression vector, pcDHMCSF11-dhfr. Plasmid, pSV2-dhfr, and pRSV-av were individually cleaved with restriction enzymes, HindIII and BamHI, and the fragments containing the DHFR gene and the LTR portion of the Rous Sarcoma Virus (RSV) were ligated together with T4 DNA ligase. After transformation of E. coli HB101 competent cells (Takara Shuzo Co., Ltd.), plasmid DNA from ampicillin-resistant colonies was examined by restriction enzyme map analysis, and the desired plasmid, pRSV-dhfr, was obtained. It contained in this order: an RSV-derived LTR (RSV-LTR); the DHFR gene; an intervening sequence and polyadenylation signal from SV40; an ampicillin resistance gene, and a replication origin (Fig. 1).

Next, a fragment containing the DHFR gene was isolated by treating the pSV2-dhfr with NdeI and BamHI, and was blunt-ended with DNA polymerase I (Klenow fragment). Also, a fragment encoding a 554-amino-acid M-CSF precursor was isolated by cleaving the plasmid, pcDHMCSF11, with SalI, and this was blunt-ended with a Klenow fragment of DNA polymerase I. These two fragments were then ligated together with T4 DNA ligase, used to transform E. coli JM109 competent cells, and the desired expression plasmid, pcDHMCSF11-dhfr, was obtained. The plasmid consisted of two transcription units for the human M-CSF and the DHFR gene, which were expressed in the same orientation under the control of the SV40 early promoter and the RSV-LTR (Fig. 1).

Cell lines and transfection. CHO cells deficient in the DHFR gene

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Abbreviations: CSF, colony-stimulating factor; M-CSF, macrophase colony-stimulating factor; rhM-CSF, recombinant human-M-CSF; CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; MTX, methotrexate; RSV, Rous Sarcoma Virus; Kd, kilodalton; PBS, phosphate-buffered saline; Na-Borate, sodium borate buffer; TFMSA, trifluoromethanesulfonic acid; RP-HPLC, reversed-phase HPLC.
(CHO-DUK-dhfr)20 were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 10% dialyzed fetal calf serum (FCS, Gibco). The cells were harvested by trypsinization and suspended in DNA injection solution (0.25 mM mannitol/0.1 M CaCl2/2H2O/0.1 mM MgSO4/7H2O/0.2 mM Tris-HCl, pH 7.2).

The expression plasmid, pDHMCSF11-dhfr, was cleaved into linear form with Cial and dissolved in the DNA injection solution. The cell suspension (2 x 10^6 cells/200 μl) and the DNA solution (30 μg of DNA/200 μl) from the TSKgel DEAE-SPW column were mixed together and the DNA was introduced into the cells with an Electric Fusion Device SSH-1 (Shimadzu) according to the manufacturer’s instructions. Electrical pulses (4.2 kV/cm^2) were applied twice at one-second intervals. The transfected cells were cultured with DMEM containing 10% dialyzed FCS, 1% nonessential amino acids (Flow Lab.), and 2% HT solution (Flow Lab.), and incubated on a 24-well plate (Corsett Corp) for 48 h. The medium was exchanged for a medium made from DMEM containing 10% dialyzed FCS and 1% nonessential amino acids that was replenished at 3- to 4-day intervals. After 10 to 14 days of incubation, 50 clones were selected from about 200 wells of transformed cells, and were further incubated in a selective medium with stepwise increasing concentrations of methotrexate (MTX; 20, 50, 100, and 400 μM). Finally, 5 clones were selected after testing for their productivity of rhM-CSF and one clone, 2.3–8, was selected for further study.

Cell culture. The transformed cells were inoculated at 1 x 10^6 cells/ml into culture flasks (10 ml/25 cm2) containing DMEM supplemented with 10% dialyzed FCS in the presence or absence of MTX (2 μM), and subcultured routinely at 4-day intervals.

For a production study, the cells were inoculated into 25 cm2 culture flasks at 1 x 10^6 cells/10 ml/flask, and cultured in MTX-free medium. Samples of the culture media were harvested daily, and frozen at −20°C until the murine bone marrow assay. The inoculated cells were also cultured for 2, 4, 6, 8, and 10 days. Then, at the indicated medium change, the medium was exchanged for fresh medium and cultured for a further two days. The rhM-CSF secreted during these two days was assayed.

Analytical gel filtration HPLC. Fifty μl of a conditioned medium of the clone 2.3–8 was put directly on a TSKgel G4000SW (Tosoh, Tokyo; 7.5 x 700 mm) HPLC column. The column was equilibrated with PBS/0.15 M NaCl/0.005% polyethylene glycol 6000 (PEG6000), and eluted with the same buffer at a flow rate of 0.8 ml/min. A mixture of thymoglobin (Sigma, St. Louis, Mo) and a standard kit (Oriental Yeast, Osaka) was used to measure the molecular weight.

Purification. The cells of the clone 2.3–8 were grown in culture flasks containing DMEM supplemented with 10% FCS (40 ml/75 cm2 or 80 ml/150 cm2), and the conditioned media (CM) were pooled. The pooled CM (650 ml) was mixed with an equal volume of 50 mM sodium borate buffer (Na–Borate), pH 8.0, containing 1.0 mM NaCl, and put on a column of ConA-Sepharose (5 x 18 cm) equilibrated in 50 mM Na–Borate, pH 8.0, containing 0.5 M NaCl. After washing the column, elution was done with the same buffer containing 0.5 mM methyl-a-D-mannoside. The eluate fractions that contained rhM-CSF were concentrated and buffer-exchanged with 50 mM Na–Borate, pH 8.0, by ultrafiltration on a YM-10 membrane (Amicon). The concentrate was then put on a TSKgel DEAE-SPW column (Tosoh, Tokyo; 21.5 x 150 mm) equilibrated with 50 mM Na–Borate, pH 8.0. The column was eluted with a NaCl gradient at a flow rate of 3 ml/min from 0 to 0.1 M for 10 min, 0.1 to 0.29 M for 75 min, and 0.29 to 1 M for 5 min. The rhM-CSF fractions (eluate at 0.23–0.29 M NaCl) were concentrated and filtered by HPLC on a TSKgel G3000SW column (Tosoh, 2.15 x 70 cm). The HPLC was run 6 times with 50 mM sodium phosphate buffer, pH 6.8, containing 0.3 M NaCl at a flow rate of 3 ml/min. The rhM-CSF fractions in the region of 114 kD were concentrated and further put on reversed-phase HPLC (RP-HPLC) using a Hiproc RP304 column (Bio Rad) equilibrated with 0.1% trifluoroacetic acid (TFA) (flow rate: 1 ml/min). Linear gradient elution of [1% TFA: AcCN (1:9)] was done as follows: 0–40% for 5 min, 40–60% for 70 min, and 60–100% for 5 min. The rhM-CSF fractions (52–53% of the eluent) were pooled and concentrated by a centrifugal concentrator after pH-adjustment with 200 mM Na–Borate, pH 8.0. The concentrate was again purified on a TSKgel DEAE-SPW column (7.5 x 70 mm) equilibrated with 50 mM Na–Borate, pH 8.0, using a linear gradient of 0–0.3 M NaCl containing 30% MeOH (v/v) at a flow rate of 1 ml/min.

We occasionally used sodium phosphate buffer, pH 7.4, instead of Na–Borate, pH 8.0, and omitted MeOH in the TSKgel DEAE-SPW HPLC.

Preparation of rabbit antiserum against IL-2/M-CSF-fused protein. An IL-2-fused M-CSF sequence that consisted of 60 amino-terminal amino acids of human IL-221 followed by the M-CSF sequence from positions 1 to 153 was expressed under the control of the irp promoter in E. coli cells in a manner similar to that described by Nishida et al.22 The fused protein was expressed as an insoluble form. After incubating E. coli with lysin buffer (50 mM Tris-HCl, pH 7.9; 25% sucrose; 1% Nonidet P-40; 0.5% deoxycholate; 2 mM DTT; 5 mM EDTA; lysozyme at 0.4 mg/ml) at room temperature for 30 min, the lyaste was extracted with 2 M urea in buffer A (25 mM Tris–HCl, pH 8.4; 2 mM dithiothreitol), and finally solubilized in 7 M guanidine–HCl in buffer A. The solubilized fused protein was further separated by centrifugation, and dialyzed against buffer A.

The dialyzed fused protein was emulsified with complete Freund’s adjuvant and about 100 to 150 μg of the protein was intradermally injected into multiple sites of New Zealand White rabbits, and followed by boosting at 2-week intervals with the protein in incomplete Freund’s adjuvant. Blood samples were collected one week after the final booster. Four rabbits developed antibody to M-CSF, and one of the four (OCT302) was used because of its high titer and high sensitivity to Western blotting.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Isoelectric focusing (IEF). SDS-PAGE was done as described by Laemmli23 after heating samples at 100°C for 5 min in the presence or absence of 2-mercaptoethanol (2-ME, final 5%). Isoelectric focusing was done with a model 1415 (Bio Rad) using an Ampholine PAG plate (Pharmacia, pH 3.5–9.5). Protein bands were silver stained with a kit (Wako Pure Chemical). We used either a preestaining kit from Bio Rad or a low molecular weight calibration kit from Pharmacia as molecular weight standards for SDS-PAGE. The pl standard for IEF was purchased from Pharmacia.

Western blotting. Electrophoresed gels were electroblotted onto nitrocellulose filters using an electrobobt apparatus (Bio Rad) according to the instruction manual. The transferred filters were first allowed to react overnight with the first antibody (OCT302), and then with peroxidase-labelled goat anti-rabbit antibody (Bio Rad) after blocking with 1% skim milk (Difco Lab.) solution. The filters were color-developed by incubating in solution containing 4-chloro-1-napthol (0.5 mg/ml, Bio Rad), hydrogen peroxide (0.01%), and MeOH (16%) in PBS.

Amino acid sequence analysis and amino acid analysis. The amino (N-terminals) sequences of the purified rhM-CSFs were identified using an automated gas-phase sequence analyzer (470A protein sequencer, Applied Biosystems). The phenyl-thiodylantoin derivatives were identified by RP-HPLC.

The amino acid analysis was done with a Hitachi amino acid analyzer after the purified rhM-CSF samples were hydrolysed at 130°C for 4 h in 6N HCl containing 1% phenol.

Deglycosylation treatment. Chemical deglycosylation, which cleaves both N-glycosidic and O-glycosidic bonds, was done with trifluoroacetic acid (TFMSA) by the method of Sojar and Bajaj24 with some modifications. Briefly, after drying the purified rhM-CSF or pyridylthethylated rhM-CSF (about 20 μg) under a vacuum, the resulting fluffy powder was treated with 25 μl of TFMSA at 4°C for 2 h. After addition of ice-cold H2O (100 μl) and pyridine (25 μl), the reaction mixture was concentrated with a Centricon 10 (Amicon), and used for SDS–PAGE.

For enzymatic deglycosylation, the purified rhM-CSF (2.5 μg) was treated at 100°C for 5 min, then incubated at 37°C for about 20 h in a 100-μl volume of the following buffers as recommended by the manufacturer: 0.1 units of Streptococcus sp. neuraminidase (Seikagaku Kogyo, Tokyo) in 20 mM sodium cacodylate buffer (pH 6.0) containing 10 mM CaCl2; 0.5 units of N-glycanase (Genzyme, Boston) in 0.1 M Na2HPO4 (pH 8.3) containing 0.13% SDS, 0.1% (v/v) Nonidet-P40, and 1 mM phenylmethylsulfonyl fluoride; 0.04 units of O-glycanase (Genzyme) in 15 mM sodium cacodylate buffer (pH 6.0). The deglycosylated rhM-CSFs were analyzed by SDS–PAGE or Western blotting.

The pyridylthethylated rhM-CSF was prepared with a combination of tri-n-butylphosphate as a reductant and 4-vinylpyridine as an alkylating reagent.
Expression of Human M-CSF in CHO Cells

Fig. 1. Construction of CHO Expression Plasmid for Native Form rhM-CSF, pcDhMCSF11-dhfr.
Plasmids were constructed as described in the text.

agent by the method of Ruegg and Rudinger as follows: about 50 μg of the purified rhM-CSF (100 μl) was mixed with 1-propanol (75 μl) and 1 M Tris-HCl, pH 8.0 (25 μl). After flushing with nitrogen, 10 μl of 1% (v/v) tri-n-butylphosphine solution in 1-propanol was added together with an equal volume of 0.5% (v/v) solution of 4-vinylpyridine in 1-propanol. The mixture was left to react at room temperature for 2 h under a nitrogen atmosphere, and purified by RP-HPLC.
Assay of M-CSF activity. M-CSF activity was measured using a murine bone marrow assay as described by Takahashi et al.24. BALB/c mouse bone marrow cells (1.5 x 10^6) were incubated in 0.5 ml of α-MEM medium containing 20% FCS and 0.3% agar at 37°C for 7 days under an atmosphere of 5% CO₂ in air. Generated colonies were counted using a dissection microscope. One unit of activity was defined as the amount of CSF required to form 1.5 colonies.

Results

Expression of rhM-CSF in CHO cells

CHO cells deficient in the DHFR gene were transformed with pcDhMCSF11-dhfr by electroporation as described in Materials and Methods. First, fifty clones were selected from about 200 wells on the 24-well plates, and further cultured in selective medium with stepwise increasing doses of MTX (0 to 400 nM). The murine bone marrow assay and the growth characteristics of these clones finally gave five clones (2.3-8; 2.3-15; 2.3-18; 2.3-20; and 2.3-42) as tentative high producer cell lines (Table I). Clone 2.3-57 was dropped due to its low growth rate. Western blot analysis of the supernatants of these 5 clones showed that the rhM-CSFs produced consisted mainly of species whose molecular mass was approximately 85 and 45 kD under nonreducing and reducing conditions, respectively (data not shown). Of these, clone 2.3-8, the activity of which was highest was selected for further investigation, then exposed to higher concentrations of MTX to 2000 nM. However, the concentrations of MTX above 400 nM did not improve the rhM-CSF productivity of the clone, and even showed growth inhibition.

As shown in Fig. 2A and B, rhM-CSF production was most efficient during the initial phase (approximately 30,000 units/10^6 cells/2 days) and gradually declined with increasing cell number.

Purification

The rhM-CSF produced by the transformant was mainly composed of species whose molecular weight was approximately 140,000 as shown by gel filtration HPLC on a TSKgel G4000SW column, but high molecular weight minor species were also present (Fig. 3). Western blot analysis showed that the molecular mass of main rhM-CSF was about 90 kD and about 45 kD under nonreducing and reducing conditions, respectively (Fig. 4). High molecular weight minor bands were also shown in the range above 150,000 as a condensed band at the top of the gel. The homogeneity as well as the recovery of purified rhM-CSFs varied from batch to batch and the purified rhM-CSFs sometimes showed molecular heterogeneity. A typical example of purification giving a homogeneously purified protein is shown in Fig. 5, and is summarized in Table II. The purified rhM-CSF was obtained with an overall yield of 6.4%, and a specific activity of 1.2 x 10^7 units/mg protein. The low recovery was due to its broad distribution on many columns, and the cutoff of rhM-CSF fractions with lower specific activities. The N-terminal sequence analysis of the purified rhM-CSF showed that the sequence was that predicted from the cDNA sequence: Glu-Glu-Val-Ser-Glu-Tyr-X-Ser-His-Met. X is predicted to be cysteine according to the cDNA sequence. The amino acid analysis did not give us

Table I. rhM-CSF Production by CHO Transforms

<table>
<thead>
<tr>
<th>Transformant</th>
<th>M-CSF Activity (units/ml)a</th>
<th>MTX 100 nM</th>
<th>MTX 400 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3-8</td>
<td>21,560</td>
<td>221,760</td>
<td></td>
</tr>
<tr>
<td>2.3-15</td>
<td>18,180</td>
<td>100,980</td>
<td></td>
</tr>
<tr>
<td>2.3-18</td>
<td>44,440</td>
<td>166,980</td>
<td></td>
</tr>
<tr>
<td>2.3-19</td>
<td>0</td>
<td>95,000</td>
<td></td>
</tr>
<tr>
<td>2.3-20</td>
<td>32,120</td>
<td>176,220</td>
<td></td>
</tr>
<tr>
<td>2.3-25</td>
<td>6,890</td>
<td>77,220</td>
<td></td>
</tr>
<tr>
<td>2.3-33</td>
<td>11,730</td>
<td>66,000</td>
<td></td>
</tr>
<tr>
<td>2.3-42</td>
<td>28,600</td>
<td>143,220</td>
<td></td>
</tr>
<tr>
<td>2.3-50</td>
<td>17,600</td>
<td>17,380</td>
<td></td>
</tr>
<tr>
<td>2.3-57</td>
<td>NDb</td>
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<td>129,360</td>
</tr>
</tbody>
</table>

a M-CSF activity by murine bone marrow assay.
b Not done.

Fig. 2. Changes in rhM-CSF Production by the 2.3-8 Cells.

A: The 2.3-8 cells were inoculated into 25 cm² flasks at 10⁶ cells/ml/flask and the culture supernatants were harvested daily, and assayed. Cell growth (×10⁶ cells/ml, —) — Concentration of rhM-CSF (×10⁶ units/ml, — — — —).

B: For evaluation of production rate, the culture supernatants were refreshed with fresh medium at the indicated days, and further cultured for 2 days. The 2-day culture supernatants were harvested for bioassay. Cell growth (×10⁶ cells/ml, —) — rhM-CSF production rate (×10⁶ units/10⁶ cells/2 days, — — — —).
Expression of Human M-CSF in CHO Cells

Fig. 3. Analytical Gel Filtration HPLC.
Fifty μl of a conditioned medium of clone 2.3-8 were injected into a TSKgel G4000SW column and eluted with PBS containing 0.15 μm NaCl and 0.005% PEG6000. Protein (absorbance at 280 nm, ———). M-CSF activity (colonies/plate, ———).

Fig. 4. Western Blot Analysis of a Conditioned Medium of Clone 2.3-8.
A conditioned medium of clone 2.3-8 was analyzed by Western blot as described in the text. 1, high-molecular-mass rhM-CSF condensed near the top of 12% gel; 2, main rhM-CSF (about 90 kD); 3, 2-ME-resistant rhM-CSF (see the text); 4, monomeric rhM-CSF (about 45 kD); 5, front marker. Molecular makers are shown on both sides.

Fig. 5. Purification of rhM-CSF Produced by the 2.3-8 Cells.
The rhM-CSF was purified from conditioned media of the 2.3-8 cells by a chromatography series described in the text. The SDS-PAGE (12% gel) profile of each step was shown under reducing conditions. The gels were silver stained. Lanes: 1 and 8, molecular markers; 2, starting conditioned media; 3, ConA Sepharose chromatography; 4, TSKgel DEAE-SPW HPLC; 5, TSKgel G3000SWG HPLC; 6, RP-HPLC; 7, re-TSKgel DEAE-SPW HPLC.

Fig. 6. Deglycosylation of the Pyridylethylated rhM-CSF.
The pyridylethylated rhM-CSF was treated with TFMS or N- glycanase as described in the text, and analyzed by SDS-PAGE (12% gel) using silver staining. Lanes: 1 and 5, molecular markers; 2, without treatment; 3, TFMSA treatment; 4, N-glycanase treatment.

Deglycosylation experiments
We did deglycosylation experiments with a purified rhM-CSF that was derived from a different batch, and consisted of a major protein band at 44 kD and a minor band at 42 kD further accompanying a 39-kD faint band on SDS-PAGE gel under reducing conditions (Fig. 7).
The results with TFMSA treatment and N-glycanase treatment of the pyridylethylated rhM-CSF are shown in Fig. 6. The TFMSA treatment resulted in the generation of two novel molecular species with molecular masses of 38 and 30 kD, while the N-glycanase treatment gave us a smear band composed of a 38-kD main band and two weak bands of 30 and 42 kD under reducing conditions.
The TFMSA treatment of the purified rhM-CSF without the pyridylethylation showed the same results (data not shown).
Table II. Summary of Purification

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>M-CSF (units)</th>
<th>Specific activity (units/mg protein)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditioned medium</td>
<td>650</td>
<td>2880</td>
<td>$6.57 \times 10^4$</td>
<td>$3.16 \times 10^5$</td>
<td>1.0</td>
<td>100</td>
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<tr>
<td>ConA Sepharose</td>
<td>67</td>
<td>169</td>
<td>$8.64 \times 10^4$</td>
<td>$5.11 \times 10^4$</td>
<td>16.2</td>
<td>132</td>
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<tr>
<td>TSKgel DEAE-5PW</td>
<td>24.5</td>
<td>23.7</td>
<td>$3.72 \times 10^5$</td>
<td>$1.57 \times 10^5$</td>
<td>49.7</td>
<td>56.6</td>
</tr>
<tr>
<td>TSKgel G3000SWG</td>
<td>4.0</td>
<td>3.06</td>
<td>$1.66 \times 10^5$</td>
<td>$5.42 \times 10^5$</td>
<td>171.5</td>
<td>25.3</td>
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<tr>
<td>RP-HPLC</td>
<td>1.88</td>
<td>0.113</td>
<td>$6.15 \times 10^5$</td>
<td>$5.44 \times 10^5$</td>
<td>1722</td>
<td>9.4</td>
</tr>
<tr>
<td>TSKgel DEAE-5PW</td>
<td>0.57</td>
<td>0.035</td>
<td>$4.23 \times 10^5$</td>
<td>$1.21 \times 10^7$</td>
<td>3829</td>
<td>6.4</td>
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</table>

* Measured by Protein Assay (Bio Rad) using BSA as a standard.

† Measured by murine bone marrow assay.

Fig. 7. Enzymatic Deglycosylation of the Purified rhM-CSF.
The purified rhM-CSF was digested with neuraminidase, N-glycanase or O-glycanase, and analyzed using Western blot analysis as described in the text. The results under reducing conditions are shown here. Lanes: 1, neuraminidase; 2, N-glycanase; 3, O-glycanase; 4, control.

not shown). We also digested the purified rhM-CSF with neuraminidase, N-glycanase, or O-glycanase (Fig. 7). The N-glycanase treatment gave us the same results as those of pyridylethylated rhM-CSF, while O-glycanase-digested rhM-CSF showed two bands with molecular masses of 41 and 37 kDa accompanying a 30-kDa minor band, the 37-kDa band being more heavily stained (Fig. 7). Neuraminidase treatment decreased the molecular mass by approximately 2 kDa, thus suggesting the presence of a terminal neuraminic acid.

Discussion

After transfection of the expression plasmid into CHO cells, which encodes for the 554-amino-acid precursor, and is expected to correspond to the native form M-CSF, a clone, 2.3–8, was isolated that was able to secrete rhM-CSF at approximately $2 \times 10^7$ units/ml after 6 days in culture. Since our previous study showed that rhM-CSF produced by pcDHMCSF11-transfected COS cells was 5000 to 10,000 units/ml after 3 days in culture, the expression level was at least 10 times higher than that of the COS cells. Available information about the expression levels of the rhM-CSF in CHO cells is limited. There seems to be only one report by Wong et al. where they described how the conditioned medium was capable of supporting the formation of murine macrophage colonies even at dilutions as high as 1:6000. Rettemier et al. reported that mouse 3T3 fibroblasts cotransfected with c-fms and the 4-kb M-CSF cDNA expressed about 5000 to 10,000 units/ml/day. Similarly, the amount of rhM-CSF secreted by mouse C127 cells transfected with BPB vectors was at the level of 5 to 15 μg/ml after 7 days in culture according to a radioimmunoassay by Manos. Although the difference between these quantitative assay methods makes it difficult to compare the results, the expression of the 2.3–8 cells is likely to be similar to those levels since the specific activities of our rhM-CSFs are approximately 1 to $6 \times 10^7$ units/mg protein.

To obtain CHO transformants with much higher expression potency, we further constructed expression plasmids other than pcDHMCSF11-dhfr: plasmids where (1) transcriptions of the M-CSF sequence and the DHFR gene are promoted by the RSV-LTR and the SV40 early promoter, respectively; (2) the transcriptional units of the M-CSF sequence and the DHFR gene are placed in an opposite orientation; and (3) the SV40 intervening sequence is moved to positions upstream from the respective two genes. However, the rhM-CSF levels secreted by transformants with these plasmids were not drastically improved and were in the range of 100,000 to 400,000 units/ml after 6 days in culture. Furthermore, when transferred to a large-scale culture system, the levels of the rhM-CSFs produced were almost the same as that of the 2.3–8 cells (M. Takahashi, unpublished results).

As already reported by many authors, the rhM-CSFs expressed by the 2.3–8 cells were also shown to be heterogeneous in their molecular weights. The molecular heterogeneity was primarily composed of two kind of species, 85-kDa main rhM-CSF and a molecular form higher than 150 kDa as shown in Figs. 3 and 4. Suzu et al. have found that the high-molecular-weight species (>150 kDa) are derived from glycosaminoglycan-containing species. The percentage by the species varied from batch to batch. The purification from media rich in high-molecular-weight species sometimes resulted in both poor recovery and low purity. In addition to molecular heterogeneity, charge heterogeneity was also shown as we experienced during the purification of M-CSF from CEM-ON cells. The results of the neuraminidase digestion suggest that the charge heterogeneity could come from the presence of terminal neuraminic acid in the carbohydrate moiety.

One of the purified rhM-CSFs consisted of two subunit species (44 and 42 kDa) with specific activity of $3.8 \times 10^7$ units/mg protein. We tried to separate these two subunit species into a single molecule by RP-HPLC after pyridylethylolation and further ion-exchange HPLC, but...
could not resolve these two molecules into separate entities with tolerable recovery. Since the appearance of two bands in the CHO rhM-CSF monomer was also reported by Halenbeck et al.,14 our case does not seem to be a rare one. The molecular mass of the two species was decreased by 6 to 12 kD by the TFMSA treatment, but it did not coalesce into a single molecule, contrary to the case of C-terminally truncated M-CSF with a C-terminus at position 153 that was also expressed as two molecular species (M. Takahashi, unpublished observation). The N- and O-glycanase treatment of the purified rhM-CSF did decrease its molecular mass, but the resulting pattern on SDS-PAGE gel was also heterogeneous (Figs. 6 and 7). Considering that we can not completely exclude such possibilities as incomplete digestion, proteolytic cleavage, or even changes in the reactivity of the deglycosylated molecules with the first antibody in Western blotting, the results of the glycosyldase-treatments seem to defy simple explanation. Since the TFMSA treatment is known to cleave both N- and O-glycosidic bonds,24 the results might suggest that there exists a case where the heterogeneity shown in main 85 kD rhM-CSF basically arises from the difference in their C-termini.

We observed the presence of 2-ME-resistant rhM-CSF species with the purified rhM-CSF as well as CM on the SDS-PAGE gels under reducing conditions as shown in Figs. 4 and 7, and this phenomenon was also shown in the SDS-PAGE of the purified, pyridylethylated rhM-CSF (Fig. 6): we recognized a smear band at the position corresponding to dimeric M-CSF. This does not seem to come from insufficient reduction.

M-CSF is a very complex molecule. Although the cDNA sequence of the human M-CSF predicts a homodimeric protein that is disulfide-bonded from the 61-kD monomeric M-CSF molecule, the forms of native M-CSFs actually isolated fluctuate from 45 to 90-kD molecules as homodimers.8,11,12,26,30 In fact, with regard to the C-terminus of mature M-CSF, there is still controversy. We purified not only a homogeneous but also a heterogeneous doublet species as a 85-kD main species. However, in spite of these molecular differences, the specific activities were in the same range (1 to 4 x 10^7 units/mg protein), and they could be used without trouble at least in in vitro studies. To further analyze in vivo the physiological roles of these heterogeneous molecular forms, we need to either develop culture conditions or transformants giving homogeneous molecular species of both the main 85-kD species and the high molecular forms, or develop more sophisticated purification procedures such as those using monoclonal antibody, which enable us to isolate each of the heterogeneous species with high recovery.

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