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Macrophage colony-stimulating factor purified from normal human urine

Amino-terminal sequence and amino acid composition

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A macrophage colony-stimulating factor (M-CSF) was purified to homogeneity from a large amount of normal human urine. Microanalysis of the N-terminal amino acid sequence up to residue 44 revealed only a single residue difference from that deduced by other workers from the nucleotide sequence of M-CSF cDNA clones. The amino acid composition of the present preparation suggested that the M-CSF which we purified possessed a structure fitting the sequence 1–190 of TPA30-1 cell M-CSF deduced by Wong et al. [(1987) Science 235, 1504–1508].

Colony-stimulating factor; Macrophage; Amino acid sequence; (Human urine)

1. INTRODUCTION

Macrophage colony-stimulating factor (M-CSF, also called CSF-1) is a protein specifically required for proliferation and functions of cells of monocyte/macrophage lineage [1].

The total amino acid sequence of human M-CSF was deduced by Kawasaki et al. [2] as well as by Wong et al. [3] from the nucleotide sequence of complementary DNA (cDNA) clones derived from human tumor cell lines. The sequence deduced by Kawasaki et al. [2] is nearly consistent with that deduced by Wong et al. [3] between position 1 and position 148. However, the latter sequence contains an additional sequence of 295 residues intervening between position 148 and position 149 of the former.

Kawasaki et al. [2] also determined the N-terminal 12 residues of human urine M-CSF and the N-terminal 30 residues of mouse L-cell M-CSF. The sequence obtained by protein sequencing exactly matched that deduced from the analysis of cDNA insofar as the first 12 residues of human M-CSF were concerned. On the other hand, mouse L-cell M-CSF differed from human MIA-PaCa-2 M-CSF at six positions in the N-terminal region, which was confirmed recently by Boosman et al. [4].

Here, we purified an M-CSF from human urine and determined its N-terminal sequence up to position 44. Except for Ser at position 37, the N-terminal sequence we determined was consistent with that deduced by the two groups of workers mentioned above. On the other hand, analysis of the amino acid composition of our preparation showed considerable differences from that predictable from the sequence deduced by Kawasaki et al. [2].

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2. MATERIALS AND METHODS

2.1. *Partial purification of M-CSF from normal human urine*

Proteins were adsorbed from fresh urine

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(10400 l) on Tokusil UR (20 kg) (Tokuyama Soda, Tokyo), extracted from the adsorbent with 1% NH_4OH and precipitated by $(NH_4)_2SO_4$ at 80% saturation. The precipitate was collected, dissolved in water, and heated at 60°C for 10 h at pH 4.5. Insoluble materials were removed by centrifugation.

After desalting, the solution was applied to a 25 × 32 cm column of DEAE-cellulofine AH (Seikagaku Kogyo, Tokyo) equilibrated with 20 mM sodium phosphate buffer, pH 4.5. The bound proteins were eluted with 20 mM phosphate/300 mM NaCl, pH 4.5. The solution was desalted and placed on a DE52 (Whatman) column (9 × 90 cm) in 20 mM phosphate/30 mM NaCl/0.05% polyethylene glycol (PEG), pH 7.4. A linear NaCl gradient (30–500 mM, 20 l) was applied, and 59% of the M-CSF activity eluted between 120 and 160 mM NaCl.

The M-CSF solution then was brought to 1 M $(NH_4)_2SO_4$ and run in 225-ml portions on a 9 × 53 cm column of phenyl-Sepharose CL-4B (Pharmacia) in 10 mM phosphate/0.15 M NaCl/0.05% PEG/1 M $(NH_4)_2SO_4$, pH 7.4. The M-CSF activity eluted between 0.8 and 0.6 M $(NH_4)_2SO_4$ on a linear gradient of decreasing $(NH_4)_2SO_4$ concentration (1.0–0 M) in phosphate/NaCl/PEG buffer (20 l).

The solution was concentrated by ultrafiltration, and 60-ml aliquots were run on a Sephacryl S300 (Pharmacia) column (9 × 90 cm) in 20 mM phosphate/0.15 M NaCl/0.05% PEG, pH 7.4 (PBS/PEG). The M-CSF eluted between 1.71 and 1.84 void volumes. All of the procedures mentioned above were carried out at 5°C.

2.2. *Final purification of M-CSF*

The above-mentioned preparation was further purified in 0.2-ml portions on a Pharmacia FPLC system equipped with two Superose-12 columns connected in series (10 × 600 mm). PBS/PEG buffer was used and 70% of the M-CSF activity emerged as a peak from 1.45 to 1.56 void volumes.

The M-CSF solution then was made 0.1% in tetrafluoroacetic acid (TFA) and loaded in aliquots (120 μg) on a μ-Bondapak C_{18} column (Waters) (3.9 × 300 mm) in a Pharmacia FPLC system. A linear gradient of increasing acetonitrile concentration (0–100%) in 0.1% TFA was applied and the M-CSF eluted at 46–48 min. The M-CSF was rechromatographed on μ-Bondapak C_{18} as above.

2.3. *Automated gas-phase Edman degradation and amino acid analysis*

An aliquot of the purified M-CSF (30 μg) was sequenced in an Applied Biosystems model 470A protein sequencer set in series with HPLC (Applied Biosystems model 120A). Aliquots (0.3 μg) of the purified M-CSF were separately hydrolyzed for 21 h in 6 M HCl at 110°C. The hydrolysate was derivatized with phenylthiocyanate and analyzed on HPLC equipped with a Pico-Tag amino-acid analysis column (Walters).

2.4. *Assay of M-CSF and other methods*

The activity of M-CSF was measured as in [5]. Serial dilution of sample solutions was performed to determine the concentration giving about half-maximal number of macrophage colonies in 7-day cultures of 10^5 mouse bone marrow cells. One unit of M-CSF corresponds to one colony in such a culture as that above.

Slab gel electrophoresis was carried out on a 10% polyacrylamide gel (7 × 8 cm) in the presence of 0.1% SDS as described by Laemmli [6], the gel being silver-stained according to Merril et al. [7]. Protein concentrations were measured by the Bio-Rad dye-binding method [8].

3. RESULTS

We purified an M-CSF from human urine to apparent homogeneity. Firstly, a partially purified preparation was obtained in 23% yield by a 5-step procedure at 5°C and stocked at −20°C without loss of activity. The preparation was further purified on the FPLC system with Superose-12 (gel-permeation) and μ-Bondapak C_{18} (reverse-phase) columns. Table 1 summarizes the results on purification of M-CSF.

The finally purified preparation (3 μg) gave a single band at an estimated molecular mass of 82 kDa when electrophoresed in the presence of 0.1% SDS. Treatment of the preparation at 37°C for 10 min with 0.5% SDS/1% mercaptoethanol converted more than 90% of the protein to a species of 36 kDa. No other distinct band was found on the gel.

An aliquot (30 μg) of the purified preparation was subjected to N-terminal sequence analysis.

Table 1

Summary of purification of macrophage colony-stimulating factor from a large amount of normal human urine

Step	Protein (mg)	M-CSF		Recovery (%)
		Units ($\times 10^{-6}$)	Units/mg ($\times 10^{-3}$)	
1. Tokusil/ammonium sulfate	46300	833	18	100
2. DEAE-cellulofine AH	21100	950	45	114
3. DE52	6110	489	80	59
4. Phenyl-Sepharose CL-4B	240	264	1100	32
5. Sephacryl S300	7.5	188	25000	23
6. Superose-12 FPLC[a]	3.0	135	45000	16
7. μ-Bondapak C_{18} FPLC[a]	0.20	21	105000	2.5

[a] Only portions of the active fraction were processed at steps 5 and 6. Data have been corrected for the portions that were not processed

Table 2

Amino acid composition of a macrophage colony-stimulating factor from normal human urine

Amino acid[a]	Content[b] (mol per mol His)	Number of residues based on					
		Sequence 1–154 (His = 4)			Sequence 1–190 (His = 5)		
		Present data	Deduced MIA-PaCa	Deduced TPA	Present data	Deduced MIA-PaCa	Deduced TPA
Ala	2.142	9	5	5	11	6	10
Asx[c]	4.357	17	18	19	22	18	22
Arg	1.250	5	6	5	6	7	5
Glx[c]	4.428	18	27	25	22	31	25
Gly	0.785	3	3	2	4	6	3
His	1.000	4	4	3	5	5	4
Ile	1.642	7	7	7	8	8	8
Leu	3.964	16	16	16	20	24	19
Lys	2.750	11	11	12	14	11	13
Met[d]	–	(4)	4	4	(5)	4	5
Phe	1.892	8	9	9	9	11	9
Pro	2.428	10	3	3	12	5	11
Ser	3.071	12	12	12	15	18	17
Thr	2.142	9	8	9	11	8	11
Tyr	1.000	4	5	5	5	6	6
Val	2.250	9	8	10	11	12	13
Trp[d]	–	(1)	1	1	(1)	2	1
1/2 Cys[d]	–	(7)	7	7	(9)	7	9
Total		154	154	154	190	190	190
M_r	17477			21558			

[a] Values are the averages of 3 independent determinations. Variation of the values was within ± 10% of each average

[b] Numbers in parentheses are the number of individual residues in the deduced sequence 1–154

[c] Combined total of free acid and acid amide

[d] Values for Met, Trp, and 1/2 Cys are taken from the paper of Wong et al. [3]

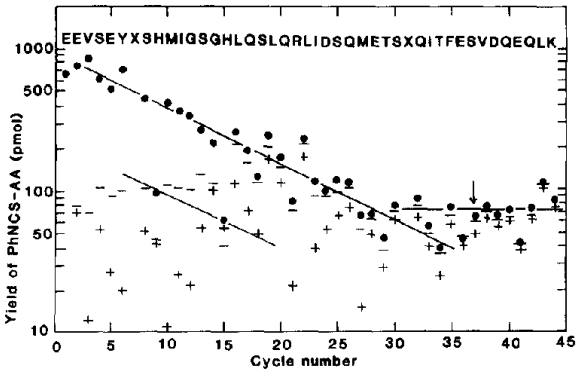

Fig.1. N-terminal amino acid sequence of human urine M-CSF. The yield of PhNCS amino acid at each cycle of automated Edman degradation is plotted vs cycle number. Small bars and plus signs represent the precycle and carry-over signals, respectively. The deduced sequence is shown in the upper part of the figure in the single-letter code. The arrow shows Ser at position 37.

The yields of phenylthiohydantoin (PhNCS) amino acids at each cycle of Edman degradation are shown in fig.1 together with precycle signals and carry-over from the preceding cycle. The N-terminus was identified as Glu. There was no other PhNCS-amino acid peak at the first cycle, which was larger than 1/60 of the PhNCS-Glu peak.

Table 2 lists the results of the amino acid analysis. The analytical values are expressed as mol individual amino acid per mol His, and the number of residues of each amino acid was calculated with respect to the integral value of His being 4 or 5. Table 2 also shows the number of amino acid residues present in the sequence deduced by other workers [2,3].

4. DISCUSSION

The advanced method described above facilitates the large-scale purification of M-CSF from normal human urine. Homogeneity of the finally purified preparation was demonstrated by SDS gel electrophoresis as well as by the N-terminal amino acid analysis.

The N-terminal amino acid sequence we determined was consistent with that deduced by other workers [2,3] except for a single difference at position 37: we found Ser instead of Phe at this position. The replacement of Ser-37 by Phe can be explained by the change of the Ser codon to a Phe codon via a single base replacement in the cDNA clone.

The amino acid composition of our M-CSF preparation most closely fits the sequence deduced by Wong et al. [3] when the integral value for each residue is calculated on the basis of the assumption that the molecule contains 5 His residues. On the other hand, the sequence deduced by Kawasaki et al. [2] does not match well with the result of our determination. Apparent discrepancy was noted with regard to the Glx, Ala and Pro contents. The results suggest that the human urine M-CSF we purified carries, at its carboxyl terminus, a part of the intervening sequence that was deduced by Wong et al. [3].

The apparent molecular mass of the M-CSF was estimated to be 36 kDa by SDS gel electrophoresis, which is about 1.7-times larger than that estimated from the amino acid composition. This difference could arise from the carbohydrate content of the molecule.

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