Characterization of human and mouse granulocyte–macrophage-colony-stimulating factors derived from *Escherichia coli*

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Human and mouse granulocyte–macrophage-colony-stimulating factors (hGM-CSF and mGM-CSF, respectively), isolated from *Escherichia coli* cells expressing the corresponding human and mouse genes, have been characterized. The observed properties of the proteins have been compared with those properties which can be deduced from the DNA sequence alone and the published properties of natural GM-CSFs. The purified *E. coli*-derived proteins were found to have the expected molecular masses, amino acid compositions and *N*- and *C*-terminal amino acid sequences. The finding of 70–90% unprocessed *N*-terminal methionine for both proteins is discussed. The four Cys residues were found to be involved in two intramolecular disulphide bonds, linking the first and third, and second and fourth Cys residues. This disulphide bond arrangement is probably the one existing in natural material, since, although not glycosylated, both *E. coli*-derived proteins showed biological activity (colony stimulating assay for hGM-CSF, and cell proliferation assay for mGM-CSF) comparable with that reported for the respective proteins purified from animal cells.

INTRODUCTION

The differentiation, activation and proliferation of the various blood cells arising from multi-potential stem cells are controlled by a group of glycoproteins synthesized by multiple cell types distributed in many tissues [1,2]. One such factor which regulates the formation of granulocytes and macrophages has been termed GM-CSF. Both the human [3] and mouse [4] proteins have been isolated, and the nucleotide sequences encoding the proteins determined [5,6]. Initial studies have indicated that these factors appear to play an important role in the control of haematopoiesis [2]; however, such studies have been limited by the unavailability of pure and well-characterized proteins. As a result of the expression of the human [7] and mouse [8] GM-CSFs in *E. coli*, sufficient material is now available for biological and structural studies. The following report describes the characterization of biologically active hGM-CSF and mGM-CSF.

MATERIALS AND METHODS

Chemicals

Protein standards for gel filtration (low M_r range) and for isoelectric focusing (pI ranges 3–10 and 2.5–6.5) were obtained from Pharmacia. All other chemicals used were of reagent grade or better.

Purification of proteins

The human and mouse GM-CSF proteins were both expressed in *E. coli* as insoluble aggregates [7,8]. The proteins were solubilized with 6 M-guanidine hydro-

chloride as described for recombinant-derived mGM-CSF [8] and renatured by equilibrium dialysis against a non-denaturing (3 M) concentration of urea (see [9] for rationale). The proteins were purified by anion-exchange chromatography using Fast Flow Q (Pharmacia) and gel filtration using Ultrogel AcA54 (LKB).

Protein determination

Molar absorbance coefficients (ϵ) for the proteins were calculated from the amino acid composition according to the procedure of Wetlaufer [10]. The values for hGM-CSF and mGM-CSF are 14.04 mM $\cdot$ cm $^{-1}$ and 10.98 mM $\cdot$ cm $^{-1}$ respectively and are based on the respective M_r values of 14650 and 14347 [5,6].

Analytical separation methods

Gel electrophoresis in the presence of SDS was carried out on 15% polyacrylamide gels using the procedure of Laemmli [11]. The proteins were visualized by either Coomassie Blue or silver staining [12]. Electrofocusing was carried out on thin-layer polyacrylamide gels (LKB Ampholine PAG plates pH 3.5–9.5) according to the protocol of the manufacturer. H.p.l.c. was performed on a system which has previously been described [13] except that the manual injector has been replaced by an automatic one (Wisp model 710B, Waters). A C $_{18}$ Radialpak μ Bondapak column was used in a Z module at a flow rate of 1 ml $\cdot$ min $^{-1}$. Solvent A was 1 g trifluoroacetic acid (h.p.l.c. grade, Pierce) dissolved in 1 litre h.p.l.c. grade water (MilliQ system, Millipore). Solvent B was pure acetonitrile (h.p.l.c. grade, Merck). The column, equilibrated with solvent A, was eluted isocratically for 5 min after sample injection, then a

Abbreviations used: hGM-CSF and mGM-CSF, human and mouse granulocyte–macrophage-colony-stimulating factor respectively.

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linear gradient of solvent B was applied (0.5%/min for protein digests, to 60% B; 1%/min for isolated peptides reduced with dithiothreitol). Samples were acidified with acetic acid prior to injection.

Reduction and alkylation of protein

Dithiothreitol (5 mM; i.e. 10 mM-SH) was added to a nitrogen-purged solution of protein (1–2 mg·ml⁻¹) which was 150 mM in Tris/HCl, pH 8.3, 2 mM in EDTA, and 3 M in guanidine hydrochloride. The solution was incubated for 1 h at 22 °C, then iodoacetamide was added to a concentration of 15 mM. After 30 min at 22 °C in the dark, a further 5 mM dithiothreitol was added and the solution dialysed against 5 mM-NH₄HCO₃ and lyophilized.

Amino acid analysis

Reduced and alkylated proteins were hydrolysed in constant-boiling HCl at 110 °C for 24, 48 and 72 h in nitrogen-purged evacuated tubes in the presence of phenol (1 mg·ml⁻¹) and 100 mM-ethanethiol. Peptides isolated by h.p.l.c. were hydrolysed for 24 h without scavengers. Hydrolysates were analysed on a Beckman 6300 amino acid analyser. The tryptophan content of the proteins was determined by ultraviolet absorption spectroscopy in the presence of 6 M-guanidine hydrochloride as described by Edelhoch [14].

Thiol and disulphide analysis

The proteins were analysed for thiol and for disulphide bonds as previously described ([15] and [16], respectively).

Location of disulphide bonds

Samples of native protein (about 5 mg/ml) were treated with CNBr (2.5 or 5 mg/ml for h and mGM-CSF, respectively) in 70% (v/v) formic acid at 22 °C for 24 h. After removal of reagent and solvent under high vacuum, samples were digested (hGM-CSF with *Staphylococcus aureus* protease V8 and mGM-CSF with trypsin and V8). Digests were examined by analytical h.p.l.c., with and without reduction [50 mM-dithiothreitol in 0.5% (w/v) NH₄HCO₃, 37 °C, 2 h] prior to chromatography. Peaks which were found to be susceptible to reduction were collected separately in a preparative run of unreduced material, then each was subjected to reduction and rechromatography. The reduced peaks thus isolated were hydrolysed and analysed on the amino acid analyser.

N-terminal and C-terminal sequence analysis

Edman degradation was performed with a gas-phase sequencer (Applied Biosystems). Identification and quantification of amino acid phenylthiohydantoin was by reversed phase h.p.l.c. with an external standard for quantification. C-Terminal sequence analysis of mGM-CSF was performed by the combined gas-liquid chromatography-mass spectrometry method [17]. C-Terminal sequence analysis of hGM-CSF was performed by digestion of native protein with carboxypeptidases A [in 1% (w/v) NH₄HCO₃, pH 7.8] and Y (in 0.1 M-pyridine acetate, pH 5.2) and direct application to the amino acid analyser with norleucine as internal standard.

Bioassays

Purified hGM-CSF was assayed for its colony-stimulating activity on a human myeloid leukaemia cell

Fig. 1. Results of SDS gel electrophoresis (lanes a–c) and isoelectric focusing (lanes d–f)

a, Standards (M_r indicated); b, hGM-CSF; c, mGM-CSF; d, standards (pI indicated); e, hGM-CSF; f, mGM-CSF.

line as described [18] (and references cited therein). Purified mGM-CSF was assayed by the cell proliferation assay [8] using the factor-dependent mouse cell line FDC-P1. In both assays, GM-CSF-dependent growth was quantified by scintillation counting of [³H]thymidine incorporated into the cells after incubation of the cells with serial dilutions of the respective GM-CSF. Activity of the recombinant GM-CSFs is expressed as the concentration of the protein which gave one-half the maximum incorporation of [³H]thymidine.

RESULTS AND DISCUSSION

Size and charge homogeneity

The results of SDS-gel electrophoresis of the *E. coli*-derived GM-CSFs are shown in Fig. 1. Single bands for both proteins are evident, with estimated M_r values similar to those predicted by the gene sequences (14650 and 14347 for hGM-CSF and mGM-CSF, respectively). No contaminating *E. coli* proteins were detected by either Coomassie Blue or silver staining. Samples pretreated with SDS in the absence of the reductant (dithiothreitol) gave similar results, indicating the absence of intermolecular disulphide cross-linked protein. The proteins behaved as monomers of expected M_r upon gel filtration on Ultrogel AcA54 in phosphate-buffered saline, pH 7.5 (results not shown).

Densitometric scanning of hGM-CSF and mGM-CSF separated by isoelectric focusing on a polyacrylamide gel stained with Coomassie Blue (Fig. 1) indicated main species constituting 97% and 93% respectively of the total protein. The minor species observed are probably charge derivatives of the GM-CSF proteins since, as aforementioned, only single bands were observed by SDS-gel electrophoresis (Fig. 1). The experimental pI values of the main species, namely 5.3 and 6.0 for hGM-CSF and mGM-CSF respectively, were very similar to those predicted from the amino acid composition using a

Table 1. Results of amino acid analyses

Values are expressed as molar ratios, with ratios predicted from the gene sequence in parentheses. Peptides 14i (95–105), 14ii (48–61), 18i (81–94), 18ii (81–94) and 18iii (110–128) from hGM-CSF and peptides 19i (73–91) and 19ii (110–120) from mGM-CSF, were isolated by h.p.l.c. (see text and Fig. 3); in these cases, the values indicated in parentheses in the Table are ratios based on the predicted sequences surrounding the Cys residues (see Fig. 2). The sequences of the peptides designated 14i etc. are indicated above. Abbreviation: n.d., not determined.

Residue	Composition (residues/molecule)								
	hGM-CSF	mGM-CSF	14i	14ii	18i	18ii	18iii	19i	19ii
Cmc*	3.82 (4)	4.16 (4)							
Asx	8.17 (8)	10.85 (11)		1.06 (1)			2.81 (3)	1.00 (1)	0.99 (1)
Thr†	10.85 (11)	15.97 (16)	2.68 (3)	1.57 (2)	0.94 (1)	0.96 (1)		2.82 (3)	1.86 (2)
Ser†	8.62 (9)	5.03 (5)	0.55 (1)		0.91 (1)	1.02 (1)		0.92 (1)	
Glx	20.91 (21)	15.06 (15)	2.00 (2)	4.00 (4)	2.00 (2)	2.00 (2)	3.00 (3)	2.03 (2)	1.03 (1)
Pro	11.53 (11)	8.83 (9)		0.93 (1)	2.96 (3)	2.87 (3)	1.87 (2)	2.94 (3)	0.97 (1)
Gly	3.32 (3)	3.08 (3)						1.00 (1)	
Ala	7.95 (8)	6.03 (6)	0.97 (1)		0.99 (1)	0.98 (1)		1.99 (2)	
Cys‡			0.74 (1)	0.78 (1)	0.84 (1)	0.64 (1)	0.66 (1)	0.24 (1)	0.84 (1)
Val	5.03 (5)	9.05 (9)					1.71 (2)		
Met	3.52 (4)	2.81 (3)							
Ile	5.63 (6)	4.79 (5)	1.19 (2)		0.97 (1)	0.86 (1)	0.74 (1)		0.94 (1)
Leu	14.20 (14)	11.19 (11)		2.97 (3)			2.89 (3)	1.00 (1)	1.00 (1)
Tyr	1.95 (2)	3.97 (4)			0.94 (1)	0.68 (1)		2.81 (3)	
Phe	4.79 (5)	6.99 (7)	0.94 (1)	0.98 (1)			1.92 (2)		1.87 (2)
His	2.97 (3)	1.01 (1)			1.95 (2)	1.84 (2)			
Hse§								0.41 (1)	
Lys	5.99 (6)	10.96 (11)			0.98 (1)	0.95 (1)	0.94 (1)		0.98 (1)
Arg	5.81 (6)	4.00 (4)		0.99 (1)					
Trp	1.80 (2)	0.75 (1)					n.d. (1)		

* The cysteine content of the proteins was estimated as carboxymethylcysteine (CMC) derived from proteins reduced and alkylated with iodoacetamide (see the Materials and methods section).

† Determined by extrapolation to zero hydrolysis time in the case of the two proteins.

‡ Determined as cystine, formed from cysteine by oxidation during hydrolysis; the results obtained have been multiplied by two so as to express the molar ratio of cysteine.

§ Determined non-quantitatively as homoserine lactone using the His colour constant.

|| Measured by ultraviolet absorption spectroscopy (see the Materials and methods section) for proteins; not determined for peptides.

simple calculation procedure based on the Henderson-Hasselbalch equation [19].

Chemical analysis

There was good agreement between the predicted and observed amino acid compositions (Table 1). The observed *N*-terminal amino acid sequence of the first 15 residues (results not shown) of both proteins was exactly as predicted from the respective gene sequences. Both proteins, however, contained 70–90% unprocessed *N*-terminal methionine. The failure of the *E. coli* methionine aminopeptidase to remove completely the initiating methionine may in principle be due to specificity considerations or simply due to saturation of the enzyme by the large amount of recombinant-produced protein [20]. The proximity of Pro in the *N*-terminal sequence Met-Ala-Pro- is probably responsible for the reluctance to cleave the initiating Met, as was proposed for the case of recombinant-derived interleukin-2 [21]. It has since been found that, under some circumstances, the initiator Met may be removed from this latter protein [22], and we find that Met is removed from *E. coli*-derived GM-CSFs to the extent of 10–30%. The proximity of Pro would thus seem to hinder Met removal rather than prevent it.

Human	1	(M)APARSPSPS	TQPWEHVNAI	21	QEARRLLNLS	RDAAEMNET
Mouse	1	(M)APTRSPITV	TRPWKHVEAI	21	KEA---LNLL	DDMPVTLNEE
Human	41	VEVISEMPDL	QEPTCLQTRL	61	ELYKQGLRGS	LTKLKGPLTH
Mouse	38	VEVVSNEFSF	KKLTCVQTRL	58	KIFEQGLRGN	FTKLKGALNM
Human	81	IASHYKQHCP	PTPETSCATQ	101	IITFESFKEN	LKDFLLVIPF
Mouse	78	TASYQTYCP	PTPETDCETQ	98	VTYADFIDS	LKTFLTDIPF
Human	121	DCWEPVQE				
Mouse	118	ECKKPVQK				

Fig. 2. Amino acid sequences of h and mGM-CSF as predicted from the gene sequence

The initiating methionine, which is removed only to a small extent (see text) is shown in parentheses. In order to align the Cys residues and to generally emphasize homology, we have introduced an unnumbered gap between residues 23 and 24 of the mouse sequence.

Carboxypeptidase A digestion of hGM-CSF showed release of only Glu and either Asn or Gln (these latter two amino acids are difficult to resolve using our analyser). Carboxypeptidase Y also released Glu and Asn/Gln in approximately a 1:1 molar ratio, followed by slower release of Val (data not shown). These results lead to the sequence assignment -Val-(Asn/Gln,Glu)-OH, which is consistent with the sequence predicted from the gene sequence, namely -Pro-Val-Gln-Glu-OH. Since the sequence -Val-(Asn/Gln,Glu)- appears once only and at the C-terminus in the predicted amino acid sequence of hGM-CSF (Fig. 2), it is reasonable to deduce that the protein, as isolated, terminates with -Val-Gln-Glu-OH, as expected. The C-terminal amino acid sequence of the mouse protein was determined by mass spectrometry to be -Val-Gln-Lys-OH (results not shown), which is the sequence expected from the gene sequence (Fig. 2).

Disulphide bonds

The four cysteine residues present in each protein were non-titratable with 5,5'-dithiobis-(2-nitrobenzoic acid), indicating the presence of two disulphide bonds per molecule. This was confirmed by the direct assay for disulphides (see the Materials and methods section), which indicated 1.97 and 2.02 disulphides per molecule for hGM-CSF and mGM-CSF, respectively.

The two major peaks of absorbance eluting during h.p.l.c. analysis of digested hGM-CSF were found to be susceptible to reduction with dithiothreitol (results not shown). Isolation of these two peaks (peaks 14 and 18 in order of elution, monitoring at 214 nm) followed by their reduction and rechromatography yielded the results shown in Fig. 3. Both of the original peaks gave rise to two new peaks, presumably via reductive cleavage of a disulphide bond: peak 14 gave rise to peaks 14i and 14ii (Fig. 3a), and peak 18 gave rise to 18i and 18iii (Fig. 3b). Peak 18i exhibited tailing, a feature quite common for peptides treated with cyanogen bromide and usually associated with partial deamidation. Amino acid analysis of the resulting fractions gave the results shown in Table 1. Fractions 14i, 14ii, 18i and 18iii correspond to residues 95-105, 48-61, 81-94 and 110-128, respectively. Fraction 18ii is seen to have essentially the same composition as 18i, consistent with its being associated with deamidation. Good agreement (Table 1) is found between the compositions of the recovered peptides and the compositions expected if disulphide bonds were to link the first and third, and second and fourth Cys residues (see Fig. 2). No other arrangement of the disulphide bonds is consistent with the amino acid compositions.

A similar result was obtained upon h.p.l.c. analysis of digested mGM-CSF (results not shown). The major component of the digest (CNBr followed by trypsin and V8) was reducible with dithiothreitol to give two new peaks (called 19i and 19ii). Amino acid analysis of these reduced peptides gave the results shown in Table 1. See Fig. 2 for the sequence of mGM-CSF. Peptide 19i corresponds to residues 73-91, liberated by the activity of trypsin at Lys-72 and of V8 at Glu-91. The failure of CNBr to cleave the sequence Met-Thr (residues 77-78) is not unusual. Peptide 19ii corresponds to residues 110-120, liberated by the activity of trypsin at Lys-109 and Lys-120. Failure of V8 to cleave the sequence Glu-Cys is attributed to steric hindrance due to the disulphide bond which was intact during the enzymic digestion. From the

Fig. 3. Results of rechromatography after reduction of disulphide-containing peptides isolated from a digest of hGM-CSF

Chromatographic conditions are given in the Materials and methods section. (a) Chromatogram obtained from peak 14, which is seen to split into two components, 14i and 14ii, which were collected separately. (b) Chromatogram obtained from peak 18, which is seen to split into two components, 18i and 18iii, which were collected separately. Peak 18i exhibits tailing and the tail of the peak was collected separately as fraction 18ii (see the text).

results of these experiments, we are led to conclude that Cys-86 is joined by a disulphide bond to Cys-119 in native mGM-CSF. Since no free thiol groups were detected, the other two Cys residues, at positions 52 and 94, must be connected by a disulphide bond. No other arrangement of disulphide bonds is consistent with amino acid compositions of peptides 19i and 19ii. This arrangement of disulphide bonds, first to third and second to fourth, is the same as that determined above for the protein of human sequence.

As discussed above, both hGM-CSF and mGM-CSF samples were, after cleavage with CNBr, digested with enzymes under mildly alkaline conditions (in a trial experiment, it had proved impossible to digest CNBr-cleaved mGM-CSF with V8 at pH 4). There exists therefore the possibility that disulphide bond scrambling took place during digestion. For the following reasons, we believe that such scrambling is unlikely. (i) Digestion took place under mild conditions [1% (w/v) NH_4HCO_3 solution, 37 °C for several hours]. (ii) No free thiol groups were present in the protein and no reducing agents were added. (iii) No evidence for other arrangements was found: several reducible peptides would have been expected if the disulphide bonds had been randomized. (iv) The same arrangement (first to third, second to fourth) was found for both samples even though the peptides isolated have quite different sequences.

Biological activity

The assays of the *E. coli*-derived GM-CSFs for biological activity, as described in the Materials and methods section, showed that both the mouse and the human proteins were highly active. The concentrations which produced half-maximal stimulation in the corresponding assays were 1.0×10^{-12} M for hGM-CSF (s.d. $\pm 0.6 \times 10^{-12}$, $n = 4$) and 6.7×10^{-12} M for mGM-CSF (s.d. $\pm 3.9 \times 10^{-12}$, $n = 8$). These values are comparable with those described for the respective proteins purified from animal cells [3,4].

CONCLUSION

For both hGM-CSF and mGM-CSF, *E. coli*-derived material was found to be similar in chemical structure and biological activity to what is expected or reported for material derived from animal cells [2,5,18]. Differences in chemical structure between natural and recombinant-derived proteins (i.e. the lack of glycosylation and the presence of initiating methionine for the *E. coli*-derived proteins) were due to differences in post-translational modification, as was found to be the case for interleukin-2 [21]. The fact that the *E. coli*-derived GM-CSFs, although lacking glycosylation and possessing an initiating methionine, are about as active as natural material, suggests that the disulphide bond arrangement determined here is probably the one existing in natural material. The availability of large amounts of pure recombinant-derived GM-CSF will hopefully allow its

clinical value in the regeneration of haemopoietic tissue and in the treatment of infectious and myeloid leukaemia to be fully assessed.

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