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Molecular Properties and Biological Activity of Human Macrophage Growth Factor, CSF-1

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A. Introduction

CSF-1 belongs to a family of colony-stimulating factors (CSF) that regulate the production of the blood cells (Metcalf 1986). CSF-1 is a specific growth and differentiation factor for bone marrow progenitor cells of the mononuclear phagocyte lineage and also promotes the proliferation of mature macrophages via specific receptors on the responding cells (Das et al. 1981; Das and Stanley 1982). CSF-1 also has a variety of stimulatory effects on the function of macrophages and monocytes. This paper summarizes the cloning of the cDNA and the genomic structure of human CSF-1, and describes properties of the macrophage growth factor that may make it a useful drug in several clinical settings.

B. Results

I. Genomic and cDNA Structure

Genomic clones for human CSF-1 were identified using DNA probes based on N-terminal sequence data of the human urinary protein. The human pancreatic carcinoma line MIA PaCa-2 was used as a source of CSF-1 protein and mRNA during induction with phorbol myristate in serum-free medium (Ralph et al. 1986a). A cDNA library was constructed from size-fractionated mRNA which was positive for a nucleotide probe and for directing the production of bone marrow growth activity in oocytes. Using a genomic probe, a cDNA clone was obtained that codes for bioactive CSF-1 upon transfection of the primate COS cell line (Kawasaki et al. 1985). CSF-1 appears to be encoded by a single-copy gene, which is about 18 kb in length and contains nine exons (Kawasaki et al. 1985; Ralph et al. 1986b), as shown in Table 1. The signal peptide is encoded by segments of exons 1 and 2. The mature polypeptide is encoded by exons 2–8.

The cDNA specifies a 32 amino-acid leader peptide followed by a 224 residue polypeptide. There are two potential N-linked glycosylation sites. At residue 59 of the mature protein, the cDNA codes for tyrosine, whereas the genomic codon is aspartic acid. This could be due to a natural polymorphism or to a reverse transcriptase error when making the cDNA library. The

Table 1. Exon-intron structure of the human CSF-1 gene

Exon	Size (bp)	Intron	Size (kb)
1	217	I	3.0
2	123	II	1.4
3	63	III	1.7
4	171	IV	4.5
5	148	V	2.0
6	131	VI	0.7
7	53	VII	0.3
8	56	VIII	0.7
9	670		

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cDNA predicts an unusual structure for a secreted protein, namely a very hydrophobic region of 23 amino acids (residues 166 to 188 of the mature protein) followed by Arg-Trp-Arg-Arg-Arg. This is typical of membrane proteins which have a transmembrane hydrophobic domain followed by three positively charged residues acting as an anchor on the cytoplasmic side (Sabatini et al. 1982). Exon 6 ends exactly after the Arg triplet, further suggesting that the gene is designed to code for a membrane protein.

II. Protein Structure and Amino Acid Homology with Murine CSF-1

Three CSF-1 molecules have been purified to homogeneity and partially sequenced: from murine L929 cells (Kawasaki et al. 1985; Ben-Avram et al. 1985; Ben-Avram 1985), human urine (Kawasaki et al. 1985), and the MIA PaCa cell line (Csejtey and Boosman 1986; Boosman et al. 1986). The sequence data to date show that the human molecules are identical to each other and to the protein predicted from the cDNA and genome (Table 2). The human and murine molecules show 74% amino acid identity over the 65 residues of the regions which have been sequenced, and are thus highly homologous.

Native human and murine CSF-1s are heavily glycosylated dimer proteins of 45000 to 70000 daltons. The unglycosylated subunits of murine and human urinary CSF-1 are reported to have a size of 14.5 daltons (Das and Stanley 1982), whereas the human cDNA predicts a polypeptide of 26 daltons. Thus, the larger translated product may have another function as a membrane-bound molecule, with intracellular protein processing or perhaps a differently spliced mRNA used to produce the secreted CSF-1. There is evidence for a cell-surface bound form of CSF-1 (Stanley et al. 1976).

III. Human CSF-1 as a Growth Factor for Human Bone Marrow Progenitors

The activity of purified human CSF-1 has been controversial. Das et al. (1981) reported that urinary CSF-1 supported the growth in agar of diffuse colonies of macrophages which were difficult to detect unless

Table 2. Homology between human and murine CSF-1 proteins

Source	Position
	1 11 21
Human urine	EEVSE Y*S
Human MIA PaCa	EEVSE Y*SHM IGSGH LQSLQ RLIDS QMETS
Human cDNA	EEVSE YCSHM IGSGH LQSLQ RLIDS QMETS
Murine L cell	× × × ×× ×
	31 41
MIA PaCa	*QITF EFVDQ EQL
cDNA	CQITF EFVDQ EQL
L cell	× × * *
	65 71 81 88
MIA PaCa	(M)(R)F(R)DN TPNA(I) A(I)(V)(Q)L QE(L)S(L)(R)**
cDNA	M R F R DN TPNA I AI V Q L QE L S L R LK
L cell	( )(×) × × ××× × × × ×

Murine protein to human cDNA homology: 48/65 = 74% identity. Human MIA PaCa and murine L-cell CSF-1 proteins were purified to homogeneity and partially sequenced (Boosman et al. 1986). Amino acid residues are shown from the N-terminus to position 88 in single letter code. *Blanks* indicate identity to the translation of the cDNA. Residues not determined (*) and residues different from the cDNA (×) are also shown. Residues from position 44 to 64 in the native protein not been determined. *Empty parentheses* indicate a tentative identification agreeing with the cDNA. *Parentheses containing* × indicate a tentative identification different from the cDNA.

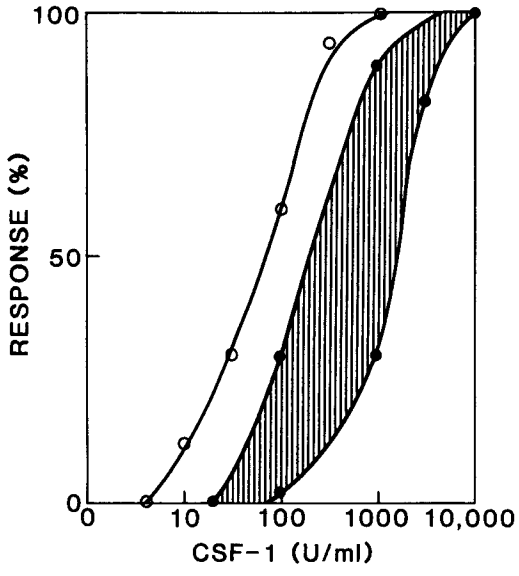

Fig. 1. Dose titration of human CSF-1 in colony formation by human and murine bone marrow cells. Bone marrow cells were cultured in agar with varying concentrations of purified human MIA PaCa CSF-1 (s.a. varying from 2×10^5 to 2×10^7 U/mg) and colonies scored at day 14 for human (range shown by *closed circles*) and day 7 for murine bone marrow cells (*open circles*) as described by Ralph et al. (1986a). Maximum colony numbers were 30–70 per 10^5 for human and 150–300 per 10^5 for mouse bone marrow cells. The variation in the human data depended on the donor response. The range of human data was seen with both partially purified CSF-1 and highly purified (about 40% pure protein, less than 0.1 ng LPS per 20000 U). Approximately 90% of the human and mouse colonies were macrophage type

persisting cells in the adherent fraction of the bone marrow population were first removed. Waheed and Shadduck (1982) found no colony-stimulating activity of human urinary CSF-1 like factor on human marrow cells, whereas Wu et al. (1981) showed that a similar factor from MIA PaCa cells stimulated human colony formation at 5% of that seen with murine cells at day 7 of culture, and at 34% by day 13 of culture. Motoyoshi et al. (1982) described a urinary protein similar to CSF-1 which stimulated granulocyte colony formation by human marrow cells through the induction of a myeloid CSF in monocytes/macrophages.

We find that purified MIA PaCa CSF-1 does induce bone marrow colonies of intensely staining macrophages (Ralph et al. 1986a). Colonies of 50 or more cells can be scored at day 14, but not day 7 of culture; they are disperse with no central concentration of cells, so that 10^5 or fewer cells should be plated. Colony formation is similar with total marrow mononuclear cells or the nonadherent fraction, and half-maximum colony formation for human progenitors occuring at 10 to 100 times the concentration required for murine cells (Fig. 1). Several of these conditions may explain why no human activity was detected in the previous studies.

IV. Effects of CSF-1 on Mature Monocytes and Macrophages

CSF-1 has direct stimulating effects on the mature monocyte and macrophage, in addition to being a growth and differentiation factor for bone marrow precursors (Table 3). It stimulates the production of prostaglandins and interferon (IFN), the intracellular killing of Candida, the production of plasminogen activator, interleukin-1, oxygen metabolites, ferritin, and a G-CSF (reviewed in Ralph et al. 1986a). Table 4 shows that CSF-1 had no immediate effect on macrophage tumoricidal activity. However, pretreatment of murine macrophages for one day with murine CSF-1 stimulated the spontaneous killing and greatly augmented the lymphokine-induced killing of TU5 sarcoma targets. The timing of the two signals was important. Pretreating macrophages with lymphokine (LK) and adding CSF-1 at the time of the cytotoxic assay did not show augmented killing (Ralph and Nakoinz 1986).

One or two days of pretreatment of macrophages with CSF-1 were optimal for the stimulation of spontaneous and LK-induced killing. Pretreatment of macrophages with 300 U/ml CSF-1 or more augmented tumor lysis induced with LK or LK plus CSF-1 in the killing assay, whereas 1200 U/ml CSF-1 was required for a large increase in spontaneous killing. The stimulatory activity in L-cell conditioned medium copurified with CSF-1 on a monoclonal immunoabsorbent column. The activity was not due to lipopolysaccharide (LPS) because the preparations had low LPS content, were active on LPS-hyporesponder C3H/HeJ macro-

Table 3. Stimulation of mature macrophage and monocyte functions by CSF-1

Function	References	
	Mouse	Human
Plasminogen activator production	Lin and Gordon 1979	
PGE production	Ralph 1984	
Ferritin production	Broxmeyer et al. 1985a	
IL-1 production	Moore et al. 1980	
Myeloid CSF production	Metcalf and Nicola 1985	Warren and Ralph 1986
IFN production	Ralph 1984	Warren and Ralph 1986
Oxygen metabolites	Wing et al. 1985	
Intracellular killing of Candida	Ralph 1984	
Tumor cytostatic activity	Wing et al. 1982	
Tumor cytotoxin		Warren and Ralph 1986
Tumor cytotoxic activity	Ralph and Nakoinz 1986	
Resist viral infection	Warren and Lee 1986	

Table 4. Tumoricidal activity of 1-day cultured but not freshly harvested macrophages is enhanced by CSF-1

Treatment		Day of assay	Cytotoxicity (%)	Effect of CSF-1
Day 0				
0		0	8	
CSF-1		0	8	No effect
LK		0	34	
LK + CSF-1		0	35	No effect
Day 0–1	*Day 1*			
0	0	1	10	
CSF-1	0	1	16	Moderate stimulation
0	LK	1	19	
CSF-1	LK	1	58	Strong stimulation
CSF-1	CSF-1	1	22	Moderate stimulation
CSF-1	LK + CSF-1	1	66	Moderate stimulation by additional CSF-1

Adherent peritoneal exudate cells from proteose peptone-injected C3H/HeN mice were tested without preincubation or after 1-day preincubation with medium or CSF-1 (300 U/ml in conditioned medium of L929 cells) for killing ^{3}H-thymidine labeled TU5 sarcoma cells at 40:1 in a 48-h assay (Ralph et al. 1982). At the time of tumor lysis assay, replicate wells received 10% v/v lymphokine (LK, 2-day concanavalin A-spleen supernate). Background radiolabel release of 7% from tumor cells alone was subtracted

phages, and were not inhibited by LPS-neutralizing polymyxin B (Ralph and Nakoinz 1986). In fact, incubation of macrophages with LPS decreased their cytotoxic activity. We have also observed protection of macrophages by CSF-1 from lytic infection by vesicular stomatitis virus (Warren and Lee 1986).

The effects of CSF-1 on human mononuclear phagocytes are just being discovered (Warren and Ralph 1986). Table 5 shows that CSF-1 treatment of human monocytes stimulates their production of IFN in response to poly I · C and the production of tumor necrosis factor and a myeloid CSF in response to LPS and PMA.

Table 5. Stimulation of human monocyte secretion of TNF, IFN, and myeloid CSF by CSF-1

Monocytes	TNF		IFN		Myeloid CSF
	LPS	LPS+PMA	pIC 10	pIC 50	LPS
Donor 1	6	12	< 6	12	0
+CSF-1	108	486	38	100	700
Donor 2	< 6	6	< 6	38	40
+CSF-1	< 6	162	400	1600	200
Donor 3	6	162	< 31	25	103
+CSF-1	18	486	38	100	377

Adherent peripheral blood mononuclear cells were incubated 3 days in DME medium containing fetal calf serum in the presence or absence of 1000 U/ml human CSF-1. CSF-1 was purified from the MIA PaCa cell line (Ralph et al. 1986a) to a specific activity of approximately 2.5×10^7 U/mg, >40% pure, <0.2 ng lipopolysaccharide (LPS)/1000 U. The cells were washed and 2.5×10^5 cells in 0.5 ml were recultured for 2 days with 1 μg/ml LPS (*Salmonella typhimurium*, Sigma), LPS+20 ng/ml phorbol myristic acetate (PMA, Sigma), or 10 or 50 μg/ml polyinosinic polycytidylic acid (pIC, Sigma). Supernatants were assayed for tumor necrosis factor (TNF), interferon (IFN), and a myeloid growth factor (myeloid CSF) as described (Warren and Ralph 1986), expressed in U/ml. The activity of CSF-1 was not blocked by LPS-neutralizing polymyxin B, and 0.1–1 ng/ml LPS (the maximum possible contamination in the CSF-1) did not stimulate monocyte production of the three factors

V. Pharmacologic Effects of CSF-1

A factor possibly identical to CSF-1 has been isolated from human urine. It induced in human monocytes the production of a G-CSF that promotes the growth of neutrophil colonies from human bone marrow precursors (Motoyoshi et al. 1982). Treatment of normal human donors with the urinary material revealed an increased production of G-CSF by peripheral blood monocytes and increased numbers of blood neutrophils and bone marrow myeloid precursors (Ishizaka et al. 1985). Studies with murine CSF-1 also demonstrated in vivo stimulation of monocytes and neutrophils as well as early pleuripotent progenitors (granulocyte/macrophage/erythrocyte/megakaryocyte-colony forming units) in mice (Broxmeyer et al. 1985 b). These clinical and preclinical results showing CSF-1 stimulation of myelopoietic events outside the mononuclear phagocyte lineage are presumably due to an indirect effect of promoting the endogenous production of G-CSF and pleuripoietins in the body. The cloning of the gene for human CSF-1 will make large amounts of this protein available for further studies.

Table 6. Expected pharmacologic uses of CSF-1

- Restore monocyte numbers (and indirectly neutrophils and other blood cells) reduced:
  - By myelosuppressive chemotherapy and gamma-irradiation for cancer and bone marrow transplantation
  - In naturally occurring anemias
- Improve resistance to infection in patients at risk:
  - Cancer, bone marrow transplantation
  - Immunodeficiencies and leukopenias
  - Elderly
  - During major surgery
- Anticancer therapy via direct stimulation of macrophages

We therefore anticipate (Table 6) that CSF-1 may find clinical utility in restoring white and red blood cell numbers that have been reduced by myelosuppressive chemotherapy or gamma irradiation for cancer treatment or bone marrow transplantation, and in naturally occurring leukopenias. CSF-1 may also have direct activating effects on mononuclear phagocytes that will improve the body's resistance to infectious diseases – viral, bacterial, and fungal – and that will stimulate the macrophages within or near tumors to destroy the neoplastic cells.

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