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PURIFICATION OF HUMAN MACROPHAGE COLONY STIMULATING FACTOR (CSF-1) FROM MEDIUM CONDITIONED BY PANCREATIC CARCINOMA CELLS

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SUMMARY: Colony Stimulating Factor-1 has been purified to apparent homogeneity from the serum-free medium conditioned by cultured human pancreatic carcinoma cells which had been induced with phorbol myristate acetate. The purification scheme consisted of sequential steps of batchwise adsorption to calcium phosphate gel, adsorption to lentil lectin-Sepharose, binding to immobilized antibodies, hydrophobic interaction chromato-graphy, and reversed-phase high-performance liquid chroma-tography. The purified glycoprotein was found to have a subunit molecular weight corresponding to the smallest of four species (approximately 40,000, 33,000, 28,000 and 23,000) which were observed when less purified preparations were examined.

CSF-1[1] is a hemopoietic growth factor which stimulates macrophage production and therefore has potential therapeutic utility. As determined by SDS-PAGE[2] in the absence of reducing agents, the molecular weight of the intact glycoprotein from human urine is approximately 46,000 (1,2,3) while the molecular weight of murine L-cell CSF-1 ranges from 47,000 to 76,000 (2,4). Irrespective of molecular weight species, treatment with reducing agent halves the apparent size, indicating that each consists of two similar disulfide-linked subunits. Analysis by SDS-PAGE of human urinary CSF-1 and murine L-cell CSF-1 which had been treated with endoglycosidases detected the presence only of N-linked carbohydrate and revealed a subunit molecular weight of approximately 14,500 for all molecules of original molecular weights up to 76,000 (2). Thus, within the range

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Abbreviations used: CSF-1, macrophage colony stimulating factor; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; cDNA, complementary desoxyribonucleic acid; HPLC, high-performance liquid chromatography.

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of molecular weights from 47,000 to 76,000 all size differences could be accounted for by differences in the levels of glycosylation.

Recently, the characterization of a full-length cDNA[3] clone obtained from CSF-1 messenger RNA isolated from cultured human pancreatic carcinoma cells was reported (5). The nucleotide sequence of this cDNA predicts a subunit polypeptide molecular weight of approximately 26,000, rather than 14,500, for human CSF-1. The purification of CSF-1 from the same cell line was developed in order to investigate this discrepancy.

MATERIALS AND METHODS

Starting Material: Pancreatic carcinoma cells (MIA PaCa-2, American Type Culture Collection, Rockville, MD) were grown to confluence in roller bottles in 10% fetal calf serum, washed once, and reincubated for 3 to 4 days in protein-free Dulbecco's Modified Eagles Medium containing 50 ng/ml phorbol myristate acetate plus 10 μM retinoic acid, as described (6). CSF-1 bioactivity was quantified by means of the radioreceptor and radioimmune assays described by Das and Stanley (7,8), using purified murine and MIA PaCa CSF-1 iodinated by Dr. Stanley. Media were filtered through Whatman No. 1 paper prior to CSF-1 purification. Polyethyleneglycol 6000 (0.01% w/w) was included in all solvents at the beginning of this study, but was later omitted when it was found to be of no benefit.

Adsorption to calcium phosphate: The method of Stanley and Guilbert (9) was adapted as follows: 50 ml of calcium phosphate gel, freshly prepared as described by Keilin and Hartree (10), were added per liter of filtered supernatant. The suspension was stirred for 1 h at room temperature and allowed to settle overnight at 4 degrees. The supernatant was decanted and discarded. The remaining supernatant was removed by centrifugation for 5 min. at 6000 x g. The recovered calcium phosphate was washed with 100 ml of 3 mM sodium phosphate, pH 6.5, and centrifuged as above. Washing was repeated twice. CSF-1 was released from the calcium phosphate by extracting with 40 ml of 50 mM sodium phosphate, pH 6.5, and centrifuging as above. The extraction was repeated twice with 25 ml portions of the same buffer. Extracts were combined and dialized overnight against water.

Affinity chromatography on Lentil Lectin-Sepharose: 1 M Tris-HCl, pH 7.5, and NaCl were added to the dialized extract from the previous step to adjust the concentrations of buffer and salt to 20 mM and 200 mM, respectively. The adjusted extract was pumped at 0.45 ml/min onto a column (2.7 cm x 4.5 cm) of lentil lectin-Sepharose (Pharmacia, Inc.) which had been equilibrated with 20 mM Tris-HCl, pH 7.5 containing 200 mM NaCl. The column effluent was monitored at 280 nm. After all the sample was applied, the column was washed with equilibration buffer until a level baseline was observed. CSF-1 was eluted by washing with equilibration buffer to which had been added 200 mM methyl-α-D-mannopyranoside. The ultraviolet-absorbing fractions (which contained most of the eluted CSF-1 bioactivity) were pooled and dialized overnight against water.

Immunoadsorption: Chromatography using a column of rat anti-murine CSF-1 which had been linked to agarose was adapted from Stanley (8). Dialized material from the previous step was adjusted to 50 mM sodium phosphate, pH 6.8, by the addition of concentrated buffer. This material was then pumped at 0.25 ml/min onto a column (2.7 cm x 7.0 cm) which had been equilibrated with 50 mM sodium phosphate, pH 6.8. After loading, the column was washed with 250 ml of equilibration buffer, and then with 0.1 M glycine-HCl, pH 2. Most of the CSF-1 activity was recovered in the 50 ml portion which was collected immediately after the pH of the effluent attained 2. The CSF-1 containing pool was adjusted to pH 7 by the addition of 1 M ammonium bicarbonate, dialyzed overnight against water, and lyophilized. The antibody column was regenerated by washing with 50 ml of 4 M potassium thiocyanate, followed by several volumes of equilibration buffer.

Hydrophobic Interaction Chromatography: Lyophilized material from the previous step was dissolved in 50 mM sodium phosphate, pH 7.0, containing 1.7 M ammonium sulfate. It was applied to a Bio-Gel TSK Phenyl-5PW column (7.5 mm x 75 mm) which had been equilibrated with the same solvent. The column was developed with a 15 minute linear gradient during which the equilibration buffer was replaced with 50 mM sodium phosphate, pH 7.0, which contained no ammonium sulfate. Flow rate throughout was 1 ml/min. Column effluent was monitored at 214 nm.

Reversed-Phase HPLC[4]: CSF-1-containing fractions from the previous step were pooled and pumped onto a Brownlee Aquapore RP 300 column (4.6 mm x 30 mm) which had been equilibrated with 0.1% aqueous trifluoroacetic acid. Proteins were eluted using a 45 min linear gradient during which the initial solvent was replaced by 60% acetonitrile containing 0.1% trifluoroacetic acid. Flow rate was 1 ml/min. The effluent was monitored at 214 nm. Fractions containing CSF-1 activity were pooled and dried under vacuum.

SDS-PAGE: Gel electrophoresis in the presence of 0.1% SDS was accomplished in 10% polyacrylamide gels as described by Laemmli (11). Reduced CSF-1 samples were prepared by boiling for 3 min in sample buffer containing 5% 2-mercaptoethanol, whereas nonreduced samples were prepared by boiling in sample buffer without 2-mercaptoethanol.

RESULTS

When calcium phosphate gel was added to the filtered supernatant from cultured MIA PaCa-2 cells as described in MATERIALS AND METHODS, essentially all of the CSF-1 activity was bound to the gel. Elution of the gel with buffer of higher ionic strength resulted in the recovery of greater total activity than in the starting material. (Table 1.) This effect was found to be reproducible over many experiments, and, as suggested by Stanley (8), is consistent with the removal of an inhibitor.

Table 1. Summary of purification of CSF-1 from serum-free medium conditioned by cultured human pancreatic carcinoma (MIA PaCa-2) cells. Results of a single experiment, in which activities were determined by radioimmune assay, are shown

Stage	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Specific Activity (U/mg)	Total Activity ($Ux10^{-6}$)	Recovery (%)
Starting Material	13,500	1,880	0.888	2,120	25.5	100
Calcium Phosphate	700	52,000	0.812	64,000	36.4	143
Lentil Lectin	95	99,500	0.178	559,000	9.5	37
Immuno-adsorption	55	235,000	0.013	18,200,000	12.9	51
Hydrophobic Interaction	---	---	---	---	5.8	23
Reversed-Phase	---	---	---	---	1.4	5

Too little protein was present in the final two steps to allow protein concentrations to be determined, thus specific activities could not be calculated.

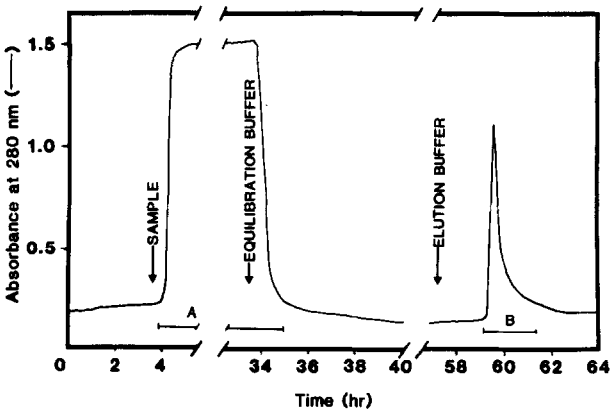

Figure 1. Affinity chromatography of calcium phosphate extracted CSF-1 on lentil lectin-Sepharose. The elution buffer consisted of equilibration buffer to which methyl-α-D-mannopyranoside had been added. Peaks were pooled as shown by the symbols (⊢——⊣). Pools A and B contained approximately equal amounts of CSF-1 activity. Only pool B was used for further purification.

The results of chromatographing the material recovered from calcium phosphate on lentil lectin-Sepharose are shown in Figure 1. In the experiment shown, approximately equal amounts of CSF-1 activity were recovered in the nonadsorbed and adsorbed fractions. This observation is consistent with that reported by Wang and Goldwasser in their study of human urinary CSF-1 (3). In this study, these proportions varied considerably from preparation to preparation and are likely to reflect differences in glycosylation of CSF-1 from one culture to the next. Reapplication of the unabsorbed fraction resulted in the recovery of only an insignificant amount of additional CSF-1 activity, indicating that overloading of the column was not the source of this effect. When the adsorbed fraction was chromatographed on the antibody column, most of the CSF-1 bioactivity was recovered in the 50 ml volume immediately following the moment that the column effluent attained pH 2. A small amount of additional activity was released by washing with potassium thiocyanate. This is in contrast to the behavior of murine L-cell CSF-1 on another column made with the same antibody. In that case, most of the CSF-1 eluted in the thiocyanate fraction, while very little was released by glycine-HCl (8). This difference is probably a reflection of lower avidity of anti-murine CSF-1 antibody for human CSF-1, and was advantageous in purifying the human factor because exposure to the chaotropic thiocyanate ion was avoided.

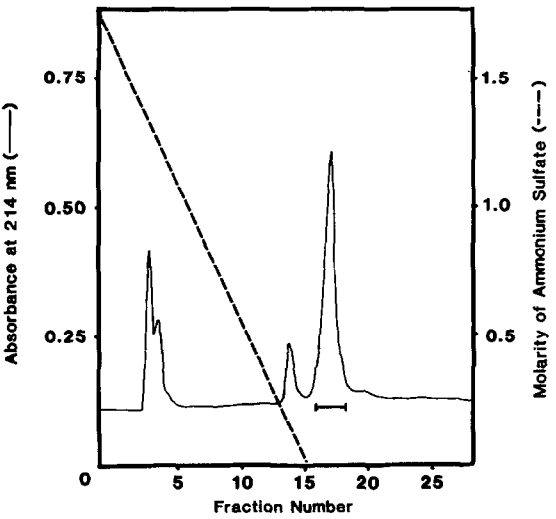

Figure 2. Hydrophobic interaction chromatography of partially purified CSF-1 on a Bio-Gel TSK Phenyl-5PW column. The sample and equilibration buffers contained 1.7 M ammonium sulfate. The gradient shown was formed by replacement of equilibration buffer with buffer which was identical, except that it lacked ammonium sulfate. Only the single pool shown by the symbol (⊢—⊣) was used for further purification.

Results of hydrophobic interaction chromatography of the glycine-eluted CSF-1 from the antibody column are shown in Figure 2. Significant levels of CSF-1 activity were detected only in the last peak. The final step, reversed-phase HPLC, produced the chromatogram shown in Figure 3. A single peak, which contained all of the recovered CSF-1 activity, was observed. The shape of this peak suggests the presence of at least

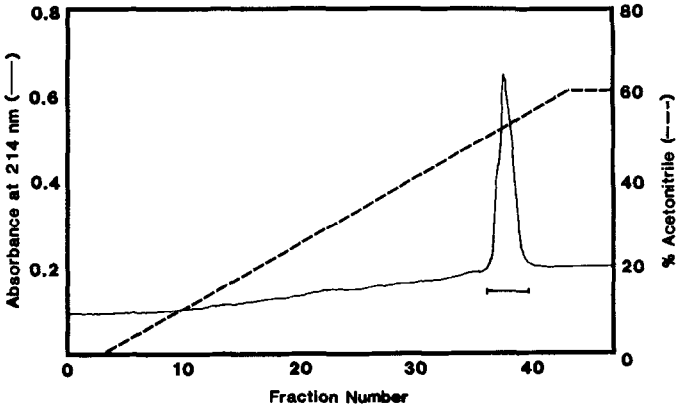

Figure 3. Reversed-phase high-performance liquid chromatography on a Brownlee RP-300 column of CSF-1 purified through the hydrophobic interaction chromatography stage. The column was equilibrated with 0.1% aqueous trifluoroacetic acid. The gradient shown was formed by replacement of the equilibration buffer with 60% acetonitrile containing 0.1% trifluoroacetic acid. CSF-1 activity was recovered in the fractions which were pooled as shown by the symbol (⊢—⊣).

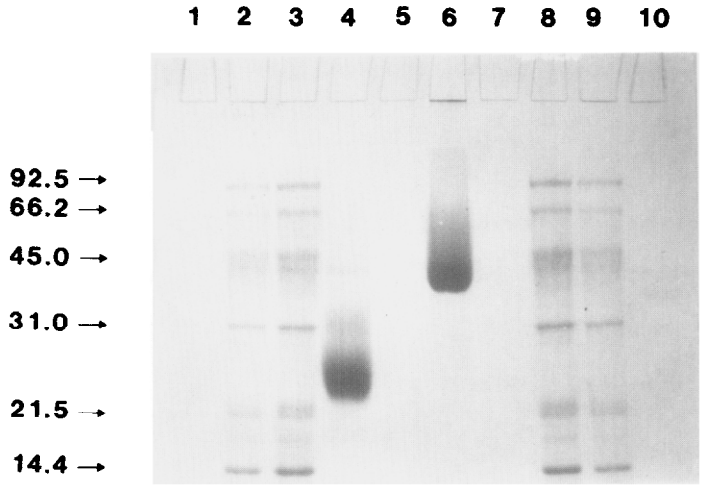

Figure 4. SDS polyacrylamide gel electrophoresis of purified MIA PaCa-2 CSF-1. Lanes 2, 3, 8 and 9 contain the following molecular weight markers: phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and hen egg-white lysozyme. Lane 4 contains CSF-1 which was reduced by treatment with 2-mercaptoethanol. Lane 6 contains CSF-1 which was not reduced.

three components, whereas the analysis of material from this peak by SDS-PAGE (Figure 4.) reveals mainly single diffuse bands before and after reduction. The higher molecular weight bands observed in the lane containing reduced CSF-1 correspond to the bands seen in less-purified material. (See DISCUSSION.) The absence of any other significant bands indicates that if contaminants are present, they must be of similar size and subunit structure. The diffuse nature of CSF-1 bands on SDS gels has been observed in all size classes of CSF-1, regardless of source, and is typical of glycoproteins. This diffuse nature may be a consequence solely of heterogeneity of the carbohydrate moieties of CSF-1 molecules, but it could mask contaminating proteins. However, structural studies (12) conclude that there is only one species of polypeptide present in HPLC-purified CSF-1, thus ruling out the presence of significant levels of contaminating proteins of any kind. This conclusion, taken together with the observation that there are no additional peaks in the reversed-phase HPLC profile, suggests that the CSF-1 was already pure following hydrophobic interaction chromatography and that the HPLC step served mainly to desalt the sample.

DISCUSSION

Several problems have made the purification of native human CSF-1 from any source difficult. CSF-1 is present at only very low concentrations in known sources, it is

polydisperse and/or heterogeneous with respect to carbohydrate (and perhaps polypeptide), it is easily lost by adsorption to surfaces, and determination of bioactivity is time-consuming. The initial concentration by batchwise adsorption to calcium phosphate solved the problem of low CSF-1 concentration in the conditioned medium. The use of multiple chromatographic steps facilitated the removal of the copius contaminating proteins. Loss of CSF-1 by adsorption was minimized by silanization of glass and plastic surfaces whenever practical. Because of the time requirements of the bioactivity determinations, it was not practical to assay all fractions recovered in each experiment. Only those fractions which earlier experiments had shown to contain the majority of the CSF-1 activity, and the nonadsorbed fraction recovered from chromatography on lentil lectin-Sepharose, were routinely assayed.

The purification results summarized in Table 1 are typical. During this study, over 30 purifications were begun, resulting in the estimated production of nearly one milligram of HPLC-purified MIA PaCa-2 CSF-1. Subsequent structural studies (12) indicated that all steps other than the lentil lectin-Sepharose step are highly reproducible and that protein recovery in the last two steps was nearly quantitative. Because of inactivation of CSF-1 in the final steps, the bioactivity assays underestimate the amount of protein recovered. The overall purification factor was estimated to be approximately 80,000 with respect to the starting material. The relationship of the CSF-1 purified in this study to the CSF-1 purified from the same source by Wu et al. (13) is not clear. Our preparations stimulate the production of macrophages exclusively, while those of Wu et al. were reported to stimulate the production of both macrophages and granulocytes.

SDS-PAGE analysis of less purified human MIA PaCa CSF-1 showed varying amounts of molecular weight species of approximately 40,000, 33,000, 28,000 and 23,000. The two larger bands have not been reported previously, but correspond closely in size to the two predominant bands seen in preparations of murine L-cell CSF-1 (2,4). They may, as appears to be the case with murine CSF-1, represent a series of more highly glycosylated versions of the same 14,500 dalton polypeptide, but this is not yet proven. This seems likely in view of the similarity of the murine and human factors. The separation of MIA PaCa-2 CSF-1 into two classes by lentil lectin-Sepharose demonstrates the heterogeneity within the carbohydrate moieties of these molecules, as was observed in human urinary

CSF-1 (3). Whether any of the MIA PaCa-2 CSF-1 molecules of higher molecular weight posses additional polypeptide is an especially interesting question in light of the subunit polypeptide molecular weight of 26,000 predicted from human cDNA and the possibility that a larger, membrane-associated version of CSF-1 might exist. The smallest band seen in less purified MIA PaCa-2 CSF-1 corresponds to the size of human urinary CSF-1 (3) and to the size of the MIA PaCa-2 CSF-1 purified in this study.

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