Journal of Chromatography, 344 (1985)339—344
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2741

Note

Purification of human urinary colony-stimulating factor by high-performance liquid chromatography

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(First received April 2nd, 1985; revised manuscript received May 24th, 1985)

Human urinary colony-stimulating factor (CSF) stimulates human monocytes to produce human granulocytic colony-stimulating factor, and mouse macrophagic colonies in agar culture [1—3]. Several groups have tried to purify human urinary CSF. In 1978, Motoyoshi et al. [4] partially purified a human urinary CSF to a specific activity of 10^6 U/mg of protein. Das et al. [5] purified human urinary CSF to a specific activity of $4 \cdot 10^7$ U/mg of protein by mouse macrophage cell receptor binding method. In 1983, Wang and Goldwasser [6] purified human urinary CSF to be an almost pure substance, with a specific activity of $1.9 \cdot 10^8$ U/mg of protein, using procedures involving Con-A Sepharose, DE-Bio Gel A, SP-Sephadex C-50, Sephadex G-150, hydroxylapatite, and a high-performance liquid chromatographic (HPLC) sizing column [6]. Our partially purified human urinary CSF has been on phase I and phase II clinical trials to granulocytopenic patients after antitumor chemotherapy [7,8]. Although the infusion of our CSF led to an earlier rise in neutrophil counts and a higher colony-stimulating activity (CSA) level in the serum, compared with non-injected leukocytopenic patients, it showed side-

0378-4347/85/$03.30

effects of lower blood pressure, fever or chilling [8]. In order to use it in clinical trials or in research on the interaction between CSF and hemopoietic stem cells, we have tried to purify CSF further using HPLC.

EXPERIMENTAL

Assay of CSA

Colony-stimulating activity was assayed in a monolayer agar culture in 35-mm plastic dishes (Falcon Plastics, Los Angeles, CA, U.S.A.). Mouse bone marrow cells were obtained from femurs of 6—8 week old C3H/He mice and used after washing with Iscove's modified Dulbecco's medium. The bone marrow nucleated cells (10^5 cells) were cultured in a monolayer in 1.0 ml containing 0.3% agar, 20% fetal calf serum, 1% bovine serum albumin (Sigma, St. Louis, MO, U.S.A.), and 10% CSF sample. CSF samples were lyophilized and diluted with culture medium containing 1% fetal calf serum, and filtered for sterility. After seven days incubation in a humidified 5% carbon dioxide atmosphere, discrete colonies containing more than 40 cells were counted under an inverted microscope. One unit of CSF was defined as the amount giving rise to one colony under the standard mouse assay system [4].

Concentration and purification of CSF

About 40 l of pooled normal human urine was concentrated by ultrafiltration (Amicon YM-10, Amicon East, U.S.A.). This was done under endotoxin-free conditions at the factory of Green Cross Corporation (Osaka, Japan). The urine concentrates contained 4000 mg of protein and a CSA ($2.0 \cdot 10^4$ U/mg of protein) when assayed by the mouse standard assay system. The urine concentrates were applied to a DEAE cellulose anion-exchange column (25× 5 cm I.D.) which was equilibrated with 0.1 *M* Tris—HCl buffer (pH 7.0) containing 0.5 mg/ml poly(ethylene glycol) 6000. The flow-rate was 50 ml/h. The column was washed with 3 vols. of the same buffer, and then eluted with a linear gradient of Cl^- ions (0.1—0.3 *M*). CSF activity was eluted from the column at ca. 0.15 *M* Cl^-. The active fractions were combined and dialysed five times against 2 l of 0.01 *M* sodium phosphate buffer (pH 6.8) containing 0.8 *M* ammonium sulphate. They were subjected to Phenyl-Sepharose CL-4B hydrophobic interaction chromatography, on a column (25× 5 cm I.D., Pharmacia, Uppsala, Sweden) that had been previously equilibrated with the same buffer. Elution was performed at 20°C with a linear salt gradient from 0.8 to 0 *M* ammonium sulphate containing sodium phosphate buffer (pH 6.8) at a flow-rate of 18 ml/h. Fractions of 10 ml were collected. The active fractions were pooled, and dialyzed five times against 5 m*M* sodium phosphate buffer (pH 7.0). They were applied to a Biogel HT hydroxylapatite column (5.0 × 1.6 cm I.D., Bio-Rad Labs., CA, U.S.A.). With a linear sodium phosphate gradient from 5 to 100 m*M*, CSF was eluted as two peaks at concentrations of ca. 40 and 80 m*M*.

High-performance liquid chromatography

All HPLC procedures were carried out at room temperature using the 655 liquid chromatograph (Hitachi, Tokyo, Japan) with two control pumps

(Hitachi) and a Rheodyne 7010 injector (Rheodyne, Berkeley, CA, U.S.A.). Column eluents were monitored on a 638-41 ultraviolet—visible variable-wavelength detector (Hitachi), and fractions were collected using a Pharmacia Frac 100 fraction collector.

The columns used were a chromatofocusing column (TSK gel DEAE-5PW, particle size 10 μm, 75 × 7.5 mm I.D., Toyo Soda, Tokyo, Japan) and a reversed-phase column (TSK gel TMS-250, particle size 10 μm, 75 × 4.6 mm I.D., Toyo Soda). A concentrated sample of 40 m*M* fractions of hydroxylapatite column chromatography was dialysed against 0.025 *M* imidazole hydrochloride buffer (pH 7.4), and applied to a chromatofocusing HPLC column, which was equilibrated with 0.025 *M* imidazole hydrochloride buffer (pH 7.4). Elution was carried out by applying 0.1% ampholine (pH 3.5, adjusted with hydrochloric acid) at a flow-rate of 0.7 ml/min, and the column was then washed with 0.5 *M* sodium chloride. Subsequently active fractions were pooled, dialysed against 10 l of distilled water, and lyophilized. The lyophilized samples, resuspended in distilled water, were applied to the reversed-phase HPLC column, which was equilibrated with distilled water containing 0.1% (v/v) trifluoroacetic acid (HPLC grade, Nakarai Chemicals, Kyoto, Japan). The samples were applied at a flow-rate of 0.5 ml/min (operating pressure, 70 bar) through a solvent line. After 30 min, the column was eluted with a 200-min linear gradient from 0 to 100% acetonitrile (HPLC grade, Nakarai Chemicals) containing 0.1% trifluoroacetic acid. The protein concentration of the column eluent was monitored by UV absorbance at 280 nm.

Determination of protein concentration

The protein concentration from steps 1—5 was determined by the method of Lowry et al. [9] with bovine serum albumin (BSA) as the standard. The protein concentration of eluents from step 6 (reversed-phase HPLC) was determined by calculating the ratio of the height of the CSF peak to the height of the BSA peak when 1, 2, 3 and 5 μg BSA were fractionated on the same column under the same conditions.

RESULTS

In attempts to find the most effective HPLC column for the purification of human urinary CSF, we first investigated an anion-exchange TSK gel QAE-2SW (IEX-520 SAE Sil) column. With a linear sodium chloride gradient or ammonium acetate gradient, CSF activity was eluted with the bulky protein peak (data not shown). Results from the anion-exchange TSK gel DEAE-5PW with a sodium chloride gradient or from the RP-CH_2OH, RP-ODS C_{18} column and the molecular sieve TSK gel G3000SW column were unsatisfactory for the same reason (data not shown). Therefore we tried to use both the chromatofocusing and the RP-TMS-250 columns. With a linear gradient of 0—100% acetonitrile, CSF activity was eluted at a concentration of 55% acetonitrile on the RP-HPLC C_4 column (Hi-Pore 304 column, particle size 5 μm, 250 × 4.6 mm I.D., Bio-Rad Labs.), and was eluted at a concentration of 45% acetonitrile on the RP-TMS 250 column (TSK gel TMS-250, particle size 10 μm, 75 × 4.6 mm I.D., Toyo Soda).

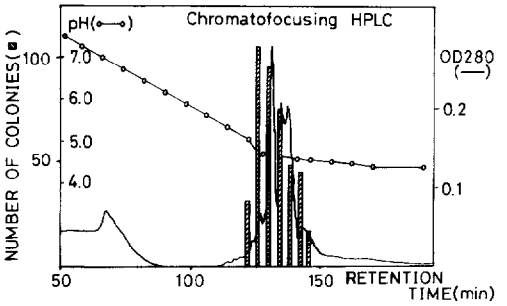

Fig. 1. Separation of human urinary CSF by chromatofocusing HPLC on a TSK gel DEAE-5PW column (75×7.5 mm I.D., Toyo Soda), which was equilibrated with 0.025 *M* imidazole hydrochloride buffer (pH 7.4). Elution was carried out by applying 0.1% ampholine (pH 3.5) adjusted with hydrochloride acid at a flow-rate of 0.7 ml/min. Active CSF was eluted between pH 5.1 and 4.5. Key: ▨ = CSF; ○ = pH; —— = protein concentration.

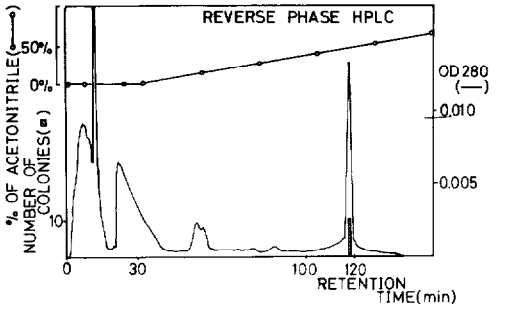

Fig. 2. Separation of human urinary CSF by reversed-phase HPLC on a TSK gel RP-TMS 250 column (75 × 4.6 mm I.D., Toyo Soda) with 0–100% acetonitrile containing 0.1% trifluoroacetic acid. Active CSF was eluted at the concentration of 45% acetonitrile. Key: ▨ = CSF; ○ = acetonitrile; —— = protein concentration.

TABLE I

PURIFICATION OF HUMAN URINARY CSF

Step	Procedure	Protein (mg)	Specific activity (U/mg of protein)	Purification factor	Yield (%)
1	Pooled normal human urine	4 000	$2.0 \cdot 10^4$	1	100.0
2	DEAE-cellulose	675	$8.3 \cdot 10^4$	4	70.0
3	Phenyl-Sepharose CL-4B	105	$4.0 \cdot 10^5$	20	52.5
4	Hydroxylapatite				
	40-m*M* fractions	12	$1.8 \cdot 10^5$	9	27.5
	80-m*M* fractions	1.1	$1.4 \cdot 10^6$	70	19.3
5	Chromatofocusing HPLC				
	pH 5.1—4.5	0.4	$1.0 \cdot 10^7$	500	5.0
6	Reversed-phase HPLC	0.003	$2.0 \cdot 10^8$	10 000	0.75

Two CSF peaks were obtained from DEAE-5PW chromatofocusing HPLC; one peak at pH 5.1—4.5, and the other at pH below 4.0. Fractions eluted at pH 5.1—4.5 had a specific activity of $1.0 \cdot 10^7$ U/mg of protein (Fig. 1). They were pooled, dialysed five times against 10 l of distilled water, lyophilized, and then resuspended in 5 ml of distilled water. They were subsequently applied to the RP-HPLC column (RP-TMS-250, particle size 10 μm, 75 × 4.6 mm I.D., Toyo Soda) and eluted with a linear gradient of 0—100% acetonitrile containing 0.1% trifluoroacetic acid. Active CSF was eluted as a single peak, which completely corresponded to a protein peak at the concentration of 45% acetonitrile with a specific activity of $2.0 \cdot 10^8$ U/mg of protein (Fig. 2). A total of 3 μg of CSF was recovered. The purification is summarized in Table I.

DISCUSSION

In our previous study, we obtained partially purified human urinary CSF of specific activity $1.0 \cdot 10^6$ U/mg of protein, with a broad but single band on non-reducing sodium dodecyl sulphate—polyacrylamide gel electrophoresis (SDS—PAGE) (Coomassie Blue R stain) [4]. Recently we found that it still had many peaks when fractionated on an HPLC TSK gel G3000SW column or an RP-HPLC column. Therefore we continued our purification procedure, using a chromatofocusing HPLC column, subsequently combined with reversed-phase column separation. We finally obtained a highly purified CSF with a single peak on RP-HPLC. It had a specific activity of $2.0 \cdot 10^8$ U/mg of protein, which was equivalent to that reported by Wang and Goldwasser [6]. They obtained a single peak of ^{125}I-labelled purified human urinary CSF on SDS—PAGE gels. Burgess et al. [10] purified CSF from mouse lung conditioned medium, which stimulated proliferation of murine granulocytic colonies. Burgess and Metcalf [11] have reviewed the nature and action of granulocyte-macrophage colony-stimulating factors. Nicola et al. [12] reported the purification of a factor inducing differentiation in murine myelomonocytic leukemia cells (WEHI-3B), which was identified as granulocytic CSF; they used phenylsilica HPLC and TSK gel permeation HPLC. Gough et al. [13] reported molecular cloning of cDNA encoding a murine granulocyte-macrophage colony-stimulating factor, which was purified from mouse lung conditioned medium.

Although a total of 3 μg of CSF was recovered, we intend to purify more materials in order to characterize further the physicochemical properties of this factor. NH_2-terminal amino acid analysis is in progress.

ACKNOWLEDGEMENT

This work was supported in part by grants in aid from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1 D. Metcalf and E.R. Stanley, Brit. J. Haematol., 21 (1971) 481.
2 H. Laukel, W.D. Gassel, H.M. Schmidt and K. Haveman, J. Cell. Physiol., 94 (1978) 21.

3 K. Motoyoshi, T. Suda, K. Kusumoto, F. Takaku and Y. Miura, Blood, 60 (1982) 1378.
4 K. Motoyoshi, F. Takaku, M. Mizoguchi and Y. Miura, Blood, 52 (1978) 1012.
5 S.K. Das, E.R. Stanley, L.J. Guilbert and L.W. Forman, Blood, 58 (1981) 630.
6 F.F. Wang and E. Goldwasser, J. Cell. Biochem., 21 (1983) 263.
7 K. Motoyoshi, F. Takaku, K. Kusumoto, Y. Miura, T. Yamanaka and K. Kimura, Jap. J. Med., 21 (1982) 187.
8 K. Motoyoshi, F. Takaku and Y. Miura, Blood, 62 (1983) 685.
9 O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
10 A.W. Burgess, J. Camakaris and D. Metcalf, J. Biol. Chem., 252 (1977) 1998.
11 A.W. Burgess and D. Metcalf, Blood, 56 (1980) 947.
12 N.A. Nicola, D. Metcalf, M. Matsumoto and G.R. Johnson, J. Biol. Chem., 258 (1983) 9017.
13 N.M. Gough, J. Gough, D. Metcalf, A. Kelso, N.A. Nicola, A.W. Burgess and A.R. Dunn, Nature, 309 (1984) 763.