A SIALOGLYCOPROTEIN-STIMULATING PROLIFERATION OF GRANULOCYTE-MACROPHAGE PROGENITORS IN MOUSE-BONE MARROW CELL CULTURES

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1. Introduction

Since the method was established for culturing macrophage-granulocyte progenitor cells in vitro [1,2], a considerable amount of work has been done to elucidate the molecular properties of the hormone-like substance (CS-factor) which is required for proliferation of progenitor cells in culture. A highly purified preparation was obtained by Guez and Sachs [3] from culture medium of a line of mouse cells (EI-cells). According to these workers, the substance is a simple protein which requires a low molecular weight cofactor for its action. In contrast, another CS-factor which was more recently purified by Stanley et al. [4] from human urine, is a sialoglycoprotein and requires no cofactors in an assay system similar to that of Guez and Sachs.

The present paper reports evidence that a CS-factor which we have partially purified from the culture medium of a subline of mouse L-cells is a sialoglycoprotein. It seems, however, that the sialic acid residue on the protein is not essential for its action, because neuraminidase treatment increased the protein's isoelectric point from 3.5–5.4 but did not significantly change its activity. Furthermore, both sialo- and asialo-CS-factor required no cofactors in our assay system.

2. Experimental

2.1. *Assay of CS-factor*

The method of assay of the colony-stimulating activity has been described [5]. Briefly, 10^5 nucleated bone marrow cells from 3 month-old male C_3H-mice were cultured in 1 ml McCoy's 5A medium containing 0.32% agar and 20% horse serum (Grand Island Biological Co.). After 7 days incubation, colonies containing more than 50 cells were counted. Addition of 0.1 ml/dish of the L-cell-conditioned medium (see below) resulted in production of about 120 colonies in the above culture, while there was no colony formation without addition of the CS-factor.

2.2. *Partial purification of CS-factor*

Mouse LP_3-cells were cultured for 5–6 days in a serum-free medium (DM-153, Kyokuto Pharmaceut. Industries Co., Tokyo) by the method of Katsuta et al. [6]. The culture medium [11] was harvested and concentrated about 20-fold by ultrafiltration (PM-10, Diaflo). After dialysis of the solution against 8 vol. saturated ammonium sulfate at 5°C, precipitates produced inside the dialysis bag were collected by centrifugation. These proteins were chromatographed at room temperature on a column of Ultrogel ACA 34 (LKB-Produkter AB, Stockholm) which had been equilibrated with 0.154 M NaCl containing 5 mM sodium phosphate buffer, pH 7.0. After elution from the column, the solution of CS-factor was concentrated to 2.5 ml by ultrafiltration and stored frozen for weeks without significant loss of the activity.

2.3. *Analytical methods*

The ultrogel column was calibrated with blue dextran (mol. wt 2 000 000), bovine serum albumin dimer (131 000), bovine serum albumin monomer (65 600), lactoglobulin (35 000), and cytochrome *c* (12 400). The latter four proteins were excluded

from the column at 1.67, 1.91. 2.15 and 2.50 void vol., respectively (av. 4 expt.).

Isoelectric focusing of CS-factor was done on a 110 ml sucrose gradient (0–50%) containing 1% carrier ampholine (LKB, pH 3–10). After electrophoresis at 500 V for 18 h, 5°C, 4 ml fractions were collected from the bottom of the column and the pH of the solutions was determined with a glass electrode. The activity of CS-factor was assayed after removal of carrier ampholites and sucrose by dialysis.

The rate of sedimentation of CS-factor was determined by the method of Martin and Ames [7]. A Hitachi model 65P ultracentrifuge was operated at 38 000 rev/min for 20 h at 10°C. Bovine serum albumin (4.5 S), lactoglobulin (3.0 S) and cytochrome *c* (1.9 S) were used for reference.

2.4. *Neuraminidase*

A highly purified preparation of neuraminidase of *Arthrobacter ureafaciens* was purchased from Nakarai Chem. Ltd, Kyoto. It contained no protease or glycosidase activities. Treatment of CS-factor with this neuraminidase (5 μg/ml) was done in 0.1 M acetate buffer, pH 5.5, for 3 h at 37°C.

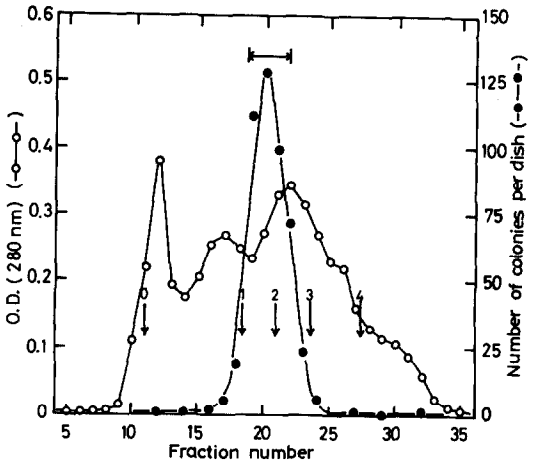

Fig.1. Chromatography of the CS-factor of LP_3-cells on an Ultrogel ACA 34 column (1.6 × 82 cm). After application of 2.2 ml sample solution, 4.2 ml fractions of the eluate were collected at a rate of 25 ml/h. Arrows represent the position of peaks for blue dextran (0), BSA-dimer (1), BSA-monomer (2), lactoglobulin (3) and cytochrome *c* (4) which were run on the same column separately. For the assay of CS-factor activity, 50 μl 100-fold dilution of each fraction was added to 1 ml cultures.

3. Results and discussion

When it was chromatographed on the Ultrogel column, the CS-factor was eluted mainly between 1.7 and 2.0 void vol. (fig.1), corresponding to a molecular weight of about 80 000 on the basis of calibration of the column with the reference proteins. However, this molecular weight estimate is by no means accurate, because glycoproteins emerge from a gel-filtration column faster than expected from their molecular weights [8] and it seems that the CS-factor is a sialoglycoprotein, as reported below.

Besides the above-mentioned peak of CS-factor activity, there was another peak of CS-factor at about 1.4 void vol. in three out of seven Ultrogel chromatography runs. The size of the peak of this larger mol. wt CS-factor differed from one experiment to another.

Isoelectric focusing of an aliquot of the smaller-molecular-weight CS-factor showed that the active principle had an isoelectric point at about pH 3.5 (av. 3 expt., range 3.4–3.6) (fig.2). The larger, mol. wt CS-factor behaved on the ampholine column in a same fashion as the smaller mol. wt CS-factor with an isoelectric point at about pH 3.5 (data not shown). This result strongly suggests that both CS-factors are sialoglycoproteins. Probably, the larger mol. wt CS-factor is a polymer of the other one.

Another aliquot of the smaller mol. wt CS-factor was treated with neuraminidase. It was found that colony-stimulating activity of the CS-factor was not significantly impaired by the enzyme treatment (fig.3), but the isoelectric point of the protein was increased to 5.4 (av. 2 expt., range 5.3–5.5). This also indicates that the CS-factor is a sialoglycoprotein. Isoelectric point of the larger mol. wt CS-factor was similarly converted to 5.5 by treatment with neuraminidase. No activity remained at around pH 3.5. This is consistent with what was observed with the smaller mol. wt CS-factor.

After isoelectrofocusing, the CS-factor was further chromatographed on a small column (1.2 × 1.5 cm) of DEAE-cellulose which had been equilibrated with 20 mM sodium phosphate, pH 7.2. Elution of the CS-factor was performed with a total of 60 ml buffer with linearly increasing concentration of NaCl up to

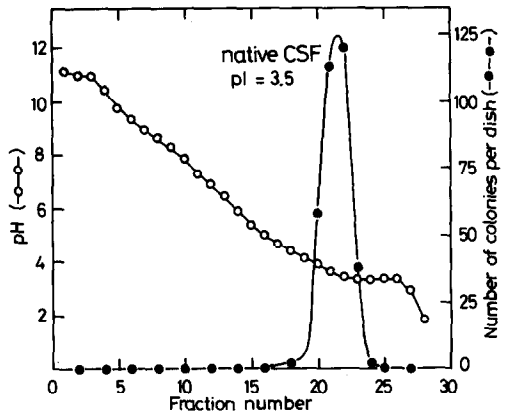

Fig.2. Isoelectric focusing of the smaller mol. wt CS-factor. The four peak fractions from the Ultrogel chromatography (see fig.1) were pooled and one-tenth was subjected to the ampholine column. After electrophoresis, each fraction was dialyzed against water and diluted to 5 ml. For the assay of CS-factor, 10 μl of each fraction was added to 1 ml cultures.

300 mM. The CS-factor appeared as a single peak between 90 mM and 120 mM NaCl. When the asialo-CS-factor was chromatographed similarly on DEAE-cellulose, all the colony-stimulating activity was found in the fractions with salt concentrations between 20 mM and 50 mM (data not shown). The sedimentation rate of these most purified CS-factors was determined by centrifugation in sucrose density-

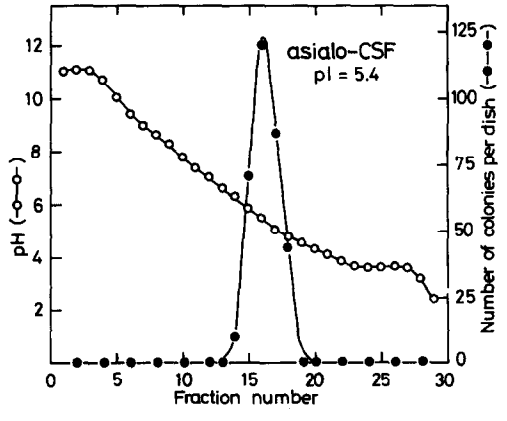

Fig.3. Isoelectric focusing of the smaller mol. wt CS-factor after treatment with neuraminidase. Conditions for electrofocusing and determination of the activity of CS-factor were the same as described for the native CS-factor (see the legend to fig.2).

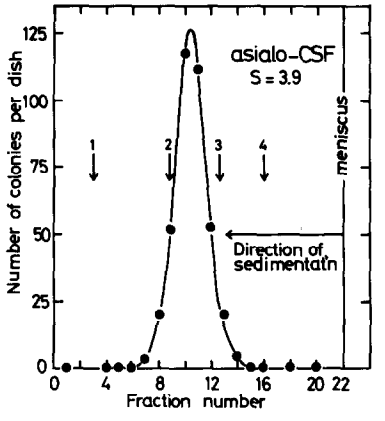

Fig.4. Determination of the sedimentation coefficient of the smaller mol. wt CS-factor by the sucrose density-gradient centrifugation method. One-tenth of the ampholine-DEAE-treated CS-factor (0.1 ml vol.) was centrifuged in a 5 ml sucrose-gradient. After centrifugation as described in Experimental, 0.23 ml fractions were collected from the bottom of the tube and 10 μl of each fraction were added to 1 ml cultures for CS-factor assay. Arrows represent the position of peaks for BSA-dimer (1), BSA-monomer (2), lactoglobulin (3) and cytochrome *c* (4) which were centrifuged similarly but on separate gradients.

gradients. The sedimentation coefficient for the native CS-factor was 4.3 S (av. 4 expt., range 4.2–4.4) (fig.4), while the sedimentation coefficient for the asialo-CS-factor was 3.9 S (av. 2 expt., range

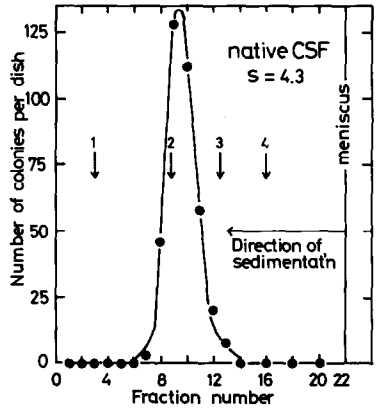

Fig.5. Determination of sedimentation coefficient of the smaller mol. wt CS-factor after treatment with neuraminidase. Details of the procedures were same as described in the legend to fig.4.

3.8–3.9) (fig.5). This result suggests that the molecular weight of the asialo-CS-factor is about 10% less than that of the native CS-factor. Although the observed decrease of the S-value may partly be due to the change of the shape of the molecule after removal of the strongly acidic group, it seems most probable that the CS-factor contains a considerable amount of sialic acid.

Apparently, no cofactors such as that reported by Guez and Sachs [3] were required for our preparations of CS-factor. No significant loss of the colony-stimulating activity occurred after exhaustive dialysis, after precipitation by ammonium sulfate, after gel-filtration chromatography, and even after isoelectro-focusing chromatography.

The extent of purification of the CS-factor by the above procedures is rather obscure. The determination of protein concentration was frustrated by the presence of phenol red at the early stages of purification and by the presence of the carrier ampholites after isoelectric focusing. Accurate determination of protein concentration was also hampered after DEAE-cellulose chromatography because of scantiness of the protein content. Further experiments are in progress and will be reported elsewhere.

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