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EFFECT OF TUNICAMYCIN ON MOLECULAR HETEROGENEITY OF COLONY STIMULATING FACTOR IN CULTURED MOUSE MAMMARY CARCINOMA FM3A CELLS

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SUMMARY

Granulocyte and macrophage colony stimulating factors obtained from cultured mouse mammary carcinoma FM3A cells showed heterogeneity in molecular size giving rise to a major component with an apparent molecular weight of 80,000 and a minor one with that of 35,000 on Sephadex G-200 column chromatography. In the presence of tunicamycin, a specific inhibitor of asparagine-linked glycosylation, the colony stimulating factor was produced normally and consisted of a single component with an apparent molecular weight of 30,000.

These data indicate that the sugar moiety is not essential for the production or activity of colony stimulating factor and that the heterogeneity in molecular size of the colony stimulating factor mainly resulted from tunicamycin-sensitive glycosylation.

INTRODUCTION

Colony formation *in vitro* by hemopoietic progenitor cells provides a useful system for investigating the molecular mechanism of hemopoiesis (1). The *in vitro* proliferation of granulocyte-macrophages in semi-solid medium is dependent on the presence of a sufficient concentration of a protein termed colony stimulating factor (CSF) (2-4). CSF has been found in the culture fluids of various cell lines and in tissue extracts.

The exact chemical structure of CSF is still unknown, and the CSFs from different sources have been found to differ in molecular weights from 70,000 for CSF from mouse L-cell conditioned medium (5,6) to 23,000 for that from mouse lung conditioned medium (7). Molecular weights of more than 100,000 have also been reported for some CSFs, but these high values may have been due to intermolecular interactions.

Abbreviations: CSF, colony stimulating factor; MEM, minimum essential medium; HEPES, 2-(N-2-hydroxyethylpiperazin-N'-yl)ethane-sulphonic acid

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Most CSFs have been found to contain carbohydrate (4-8), and it has been suggested, though not yet confirmed, that the heterogeneity in size and charge of CSF molecules is mainly due to heterogeneity in glycosylation (4-8).

In the present study, we examined the effect of tunicamycin, a specific inhibitor of asparagine-linked glycosylation (9-11), on the production and heterogeneity in size of CSF produced by cultured mouse mammary carcinoma FM3A cells.

MATERIALS AND METHODS

Cells and cell culture

FM3A cells were originally established from a spontaneous mammary carcinoma in a C3H/He mouse (12). Mutants TM5 and TM7, which are resistant to tunicamycin, were isolated from FM3A by selective culture in medium containing tunicamycin at 1μg/ml. Both mutants showed phenotypic alteration in ability to incorporate sugars into intracellular macromolecules.

The cells were cultured at 37°C in a modified Eagle's minimum essential medium (MEM) (13) supplemented with 0.1% Bactopeptone (Difco) and 2% fetal calf serum (GIBCO).

Assay of CSF

CSF was assayed as described previously (6). One unit of CSF represents the activity for producing one granulocyte-macrophage colony, when 10^5 nucleated bone marrow cells of male C3H mice are seeded into a 35 mm petri dish containing 1 ml of semi-solid medium in the presence of 20% conditioned medium from FM3A cells. Granulocyte-macrophage colonies were counted under a microscope after culture for 7 days.

Chromatography of CSFs on a Sephadex G-200 column

FM3A cells were cultured in serum-free medium with or without tunicamycin at 1μg/ml for one day. The culture fluids were dialyzed at 4°C against 2mM HEPES,pH 7.4, containing 0.09% NaCl and concentrated 10-fold in a rotary evaporator. These preparations were chromatographed at 4°C on a Sephadex G-200 column (1 x 40 mm) equilibrated with MEM plus 20mM HEPES, pH 7.4, and the CSF activity and protein content of each fraction were determined.

RESULTS AND DISCUSSION

1. Cell growth and rate of production of CSF from FM3A cells

First, the relation between production of CSF and growth of the cells was examined. As shown in Fig. 1, the rate of production of CSF increased progressively during the exponential growth phase and was maximal when the cells entered the stationary phase of growth. These results suggested that the rate of production of CSF is regulated by the growth phase of the cells.

In subsequent experiments production of CSF was examined with cells in the early-stationary phase when CSF production was maximal.

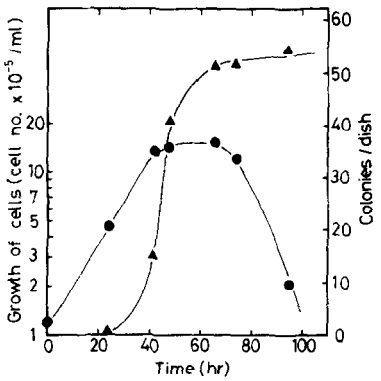

Fig. 1. Time course of production of CSF in conditioned medium of FM3A cells. The cells were inoculated into 5 ml of fresh medium at 1 x 10^5 cells/ml. The conditioned medium obtained after culture for appropriate times was dialyzed against fresh medium and assayed. Symbols: —▲—, CSF activity; —●—, cell growth.

2. Effect of tunicamycin on the production of CSF by FM3A cells

Tunicamycin at 0.2μg/ml caused more than 95% inhibition of glycosylation of asparagine-linked glycoproteins in FM3A cells with slight inhibition of protein synthesis (unpublished data). However, the production of CSF by the cells was not inhibited by a much higher concentrations of tunicamycin (Table 1). Therefore, tunicamycin-sensitive glycosylation of CSF is not essential for the secretion of CSF from the cells or for CSF activity.

3. Effect of tunicamycin on molecular heterogeneity of CSF produced from FM3A cells

The CSFs produced from FM3A cells were examined by Sephadex G-200 column chromatography. As shown in Fig. 2, the CSFs consisted of a major and a minor component with apparent molecular weights of 80,000 and 35,000, respectively.

The CSFs produced by the tunicamycin-resistant mutants TM5 and TM7 cells showed the same heterogeneity in molecular size as those produced by FM3A cells, but the relative amount of the major component to the total CSFs produced was significantly more with TM7, and less with TM5 than with FM3A cells. These differences may reflect differences in glycosylation, since TM7 cells showed an increase by about 2-fold and TM5 cells showed a decrease by about one half in the capacity to incorporate sugars into cell-bound macromolecules as compared with FM3A cells (data not shown).

Table 1. Effect of tunicamycin on production of CSF in FM3A cells.

Tunicamycin	Colonies/dish		
	Exp. I[a]	Exp. II[b]	Exp. III[c]
0 μg/ml	24	66	12
0.2	34	78	23
0.5	32	71	18
1.0	41	79	18

The conditioned media were dialyzed against fresh culture medium and the activity of CSF was assayed as described in MATERIALS AND METHODS. Values represent means of duplicate assays.

a) Cells were inoculated at 1.1 x 10^6 cells/ml and allowed to produce CSF for 1 day.
b) Conditions were as for a) except that cells were inoculated at 2.2 x 10^6 cells/ml.
c) Conditions were as for a) except that cells were inoculated at 1.9 x 10^6 cells/ml and serum-free medium was used.

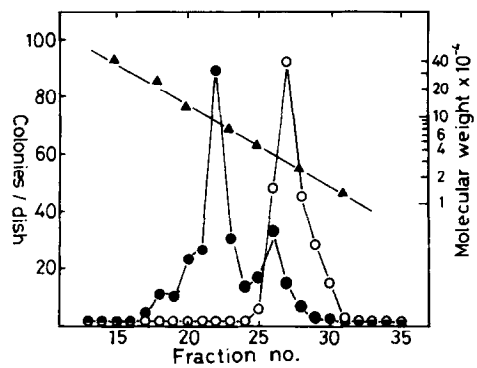

Fig. 2. Gel filtration of CSFs produced in the presence and absence of tunicamycin. For details, see MATERIALS AND METHODS. The marker proteins used for calibration of the column were ferritin, catalase, aldolase, bovine serum albumin, ovalbumin, chymotrypsin and cytochrome c. Symbols: —●— , CSF produced in the absence of tumicamycin; —○— , CSF produced in the presence of tunicamycin; —▲— , calibration curve for molecular weight determination.

In contrast, the CSF produced by FM3A cells in the presence of tunicamycin had a lower molecular weight of 30,000 and was homogeneous, as shown in Fig. 2. The CSF produced in the presence of tunicamycin had no affinity to Concanavalin A-Sepharose, indicating that it was devoid of sugars, but the CSF produced in the absence of tunicamycin bound to Concanavalin A-Sepharose (data not shown). These results show that the heterogeneity in molecular size of CSF is mainly due to differences in glycosylation of a single polypeptide

and that inhibition of glycosylation by tunicamycin resulted in production of a single homogeneous component of the CSF.

Analogous with the case of CSF, interferons from various sources were reported to be heterogeneous in molecular weight (14) and charge (15), and this heterogeneity was recently shown to be mainly due to differences in carbohydrate moieties (16-19). The heterogeneity in size of CSF resembles that of interferon in that both glycoproteins consist of a major component with a large molecular weight and a minor one with a small molecular weight. We still do not know whether these heterogeneities have any biological significance or whether they merely reflect differences in glycosylation depending on the culture conditions. The biological significances of glycosylation of CSF and interferon require further investigation.

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