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Long-Term Cultivation of a Human Colony-stimulating Factor-producing Cell Line in a Protein-free Chemically Defined Medium[1]

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ABSTRACT

To examine whether a human colony-stimulating factor (CSF)-producing cell line, T3M-1, can propagate and secrete CSF without protein supplements, long-term cultivation of the cells was carried out in a protein-free chemically defined medium. By the use of stepwise decreases in the fetal bovine serum concentration, continuous growth of T3M-1 cells has been established in a protein-free F-10 medium. The cells designated T3M-1-T2 have been propagated in this medium for 5 years. The population-doubling time of the cells is about 30 hr. Addition of serum stimulated the cell growth (population-doubling time, 17 hr) but did not increase the saturation density. The cells secreted large amounts of CSF (2000 colonies stimulated by 1 ml of the conditioned medium). Addition of serum to the culture increased CSF activity in the conditioned medium (3400 colonies/ml). The results showed that a human cancer cell line, T3M-1-T2, could be propagated in a protein-free chemically defined medium and secrete large amounts of CSF. The cells will serve as an excellent model for better understanding of the cell growth and production of CSF in the absence of any serum contamination.

INTRODUCTION

Learning how cells of multicellular animals control their proliferation rates has long been a key objective of experimental biology. Nutritionally simple microbes offer several advantages for biosynthetic studies on animal tissues. Compared to *Escherichia coli*, our knowledge of the structural organization and biochemistry of eukaryotic cells is still pitifully meager. The greater potential of tumor cells to yield cell lines has led to the belief that all cells that grow as cell lines perhaps do so because they have acquired many of the essential properties of the cancer cell by mutation(s). In general, the growth in culture requires not only a well-defined collection of amino acids and vitamins but also depends upon protein factors present in blood serum. In the absence of such growth-promoting factors, most cells enter a resting state of G_1 phase of the cell cycle, with a substantial reduction of cell numbers or complete cell death within a few days. The mechanisms by which such factors affect cell functions have not been understood fully. Among these factors, even the effects of insulin on cell growth and functions have not been clarified (15). The complex nature of such factors represents an undefined variable when assessing the biochemical changes within the cell during proliferation or differentiation (7, 15). To circumvent this problem, long-term cultivation of human functional cells was performed in a protein-free chemically defined medium. This protein-free culture may provide advantages similar to bacterial cultures, especially for genetic research of cells, because mutations to auxotrophy in this system may be possible in the absence of serum factors.

MATERIALS AND METHODS

Cell Culture. A human CSF[3]-producing cell line, T3M-1, was established from a squamous cell carcinoma of the oral cavity as described previously (14). The cells have been continuously propagated in F-10 nutrient medium supplemented with 10% FBS (Flow Laboratories, Inc., Rockville, MD), penicillin (100 units/ml), and streptomycin (100 µg/ml). Triple-distilled water was used to prepare medium. Stock cultures were grown in flasks (Falcon No. 3013; 25-sq cm surface area) in 8 ml of medium in a fully humidified atmosphere of 5% CO_2 in air at 37°. Cells were harvested with 0.25% trypsin (Grand Island Biological Co., Grand Island, NY) and 0.02% EDTA in calcium- and magnesium-free balanced salt solution as described (14). After successive treatment of the cells with trypsin and then EDTA solution, cells suspended in EDTA solution were diluted with F-10 medium which contained large amounts of calcium.

Growth Curve. The cells of passage 57 in a protein-free F-10 medium were studied to estimate the population-doubling time. The initial cell number was 1×10^5 cells/dish for a total 30 dishes (Falcon No. 3001; 35 × 10 mm). The cells were grown in 2 ml of F-10 medium without supplements. A cell count was taken each day in 2 dishes, and the medium was changed every day.

Morphological Examination. Cultured cells were photographed without stain by using a phase-contrast microscope or were stained with Giemsa dye.

Chromosomal Analysis. For chromosomal preparations, the cells in exponential growth phase were treated with Colcemid (0.2 µg/ml; Grand Island Biological Co.) for 2 to 3 hr at 37° and then with hypotonic KCl solution (0.5%) for 20 min. The cells were fixed for 10 min in methanol:acetic acid (3:1). After 3 changes of fixative, the cells were dropped onto wet slides, dried over an alcohol lamp, and stained wtih conventional Giemsa for counting the number of chromosomes. For identification of chromosomes, the Q-banding technique was used as described (14).

Assay for CSF. CSF activity was assessed by a modification of the method originally described by Bradley and Metcalf (3). In the present studies, nonadherent cell fractions of bone marrow cells were obtained by sternal puncture with heparinized syringes from normal volunteers who gave informed consent. Nonadherent cells were prepared by the method described previously (12, 14), *i.e.*, after washing and removal of erythrocytes by hypotonic lysis, the bone marrow cells were incubated in glass dishes to remove adherent cells. A constant number (2×10^5) of nonadherent nucleated cells was cultured in a single layer in 1.0 ml of the supplemented McCoy's Medium 5A (Grand Island Biological Co.) containing 0.3% agar (Difco Laboratories, Detroit, MI), 15% FBS (Flow Laboratories, Inc.), and varying concentrations of culture medium or control medium. In the control cultures, conditioned medium from GCT cells (5) (Grand Island Biological Co.) was used as a standard CSF. For studies of dose response, the original samples were diluted with McCoy's

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[3] The abbreviations used are: CSF, colony-stimulating factor; FBS, fetal bovine serum.

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Medium 5A. After 10 days of incubation in a fully humidified atmosphere of 7% CO_2 in air, discrete colonies containing 50 or more cells were counted with an inverted microscope. One unit was defined as one colony from 2×10^5 cells. Protein was assayed by the method of Lowry *et al.* using bovine serum albumin as a standard (9).

RESULTS

Adaptation of Cells to a Protein-free Medium. Stationary culture of T3M-1 cells in Ham's F-10 medium containing 10% serum was subcultured at a 1:3 ratio into F-10 medium with 2% FBS. An attempt to subculture the cells directly into serum-free medium resulted initially in some cellular proliferation but no sustained growth. Stepwise decreases in the FBS concentration at weekly or biweekly intervals were then used until the serum was eliminated from the medium. This total time interval could be as short as 3 months, depending on the use of high seedings for subcultures.

Characterization of Adapted Cells. The cells established in protein-free medium, T3M-1-T2, were morphologically similar to the original T3M-1 cells. Most of the cells had a large round nucleus with prominent nucleoli (Fig. 1). A kinetic study on the *in vitro* growth of T3M-1-T2 cells in a protein-free medium is shown in Chart 1. The cells used for these experiments were the 72nd transfer generation cells, completely adapted to the protein-free medium and growing vigorously. The experimental cultures were initiated with an inoculum of 1×10^5 cells and incubated in a CO_2 chamber at 37°. The growth curve was obtained by counting the number of cells per dish as a function of the incubation time. From the curve in Chart 1, the population-doubling time was estimated as 30 hr. Addition of serum stimulated the growth of the cells (population-doubling time in serum-supplemented culture, 17 hr). However, the saturation density in protein-free cultures exceeded that in serum-supplemented culture (Chart 1).

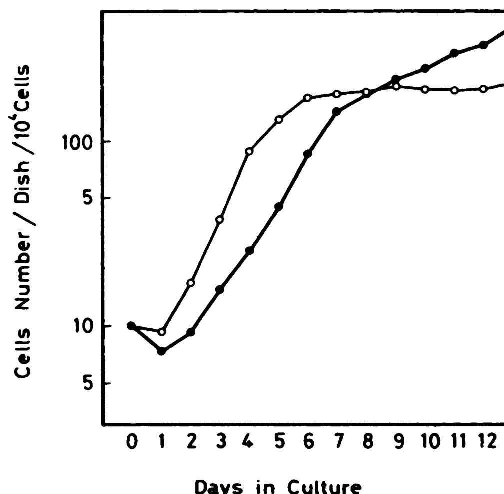

Chart 1. Growth curve of T3M-1-T2 cells in a protein-free medium. The cells of passage 72 were grown in 2 ml of protein-free F-10 medium. The medium was changed every day. *Points*, mean value from 2 dishes. ●, without serum; O, with 10% FBS.

Table 1
Chromosomal pattern of T3M-1-T2 cell line

Passage	Date of examination	Chromosome no. distribution								Total cells counted
		59	60	61	62	63	64	65	66	
256	May 21, 1983	1	0	1	4	8	24	8	4	50

The modal chromosome number of T3M-1-T2 cell line was 64 in the 256th passage, with considerable scattering in counts (Table 1). The Q-banding technique revealed the presence of most of the common markers which had been identified in the parent T3M-1 cell line (Fig. 2) (14). Normal Y chromosome with a brilliant fluorescent body and a Y translocated onto No. 3 chromosome (M2 chromosome) (14) were found in T3M-1-T2 cells.

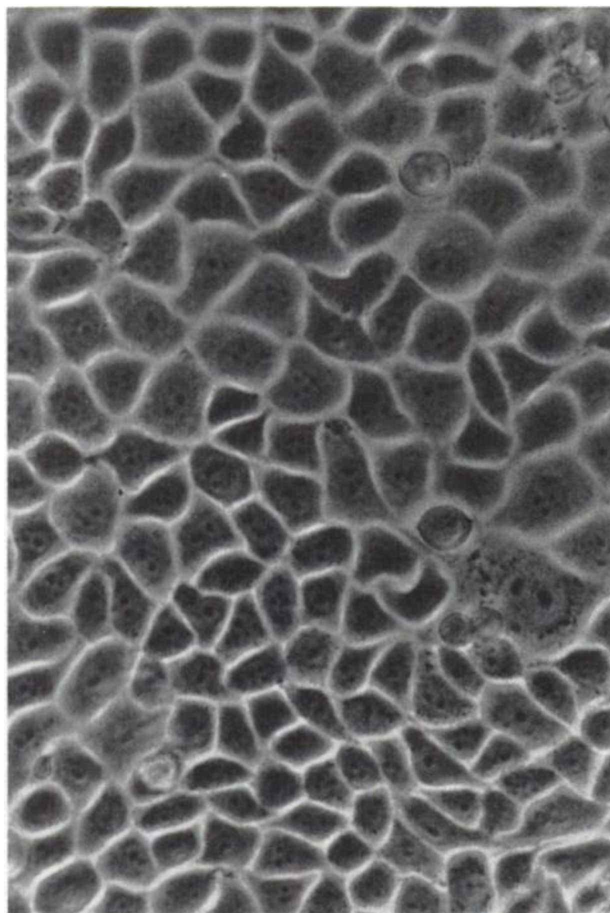

Fig. 1. Morphological features of T3M-1-T2 cells grown in a protein-free F-10 medium. Phase-contrast microscopy, × 560.

CSF Production in T3M-1-T2 Cells in Protein-free Medium. Confluent culture of T3M-1-T2 cells established in a protein-free F-10 medium was incubated in F-10 medium with or without 10% FBS. Medium was harvested after 1 week of incubation and assayed for CSF activity. Representative data on the production of CSF by the cells are shown in Chart 2. Conditioned medium from the protein-free culture stimulated colony formation of human bone marrow cells in a dose-dependent manner. The titer of CSF activity in the medium was approximately 2,000 units per ml of medium. Because of the low protein concentration of the medium (77 μg/ml), specific activity was increased to 25,974 units per mg of protein. Higher CSF activity (3,400 units/ml) was demonstrated in the conditioned medium, when the culture was incubated in serum-supplemented medium. However, specific activity was decreased to 636 units per mg of

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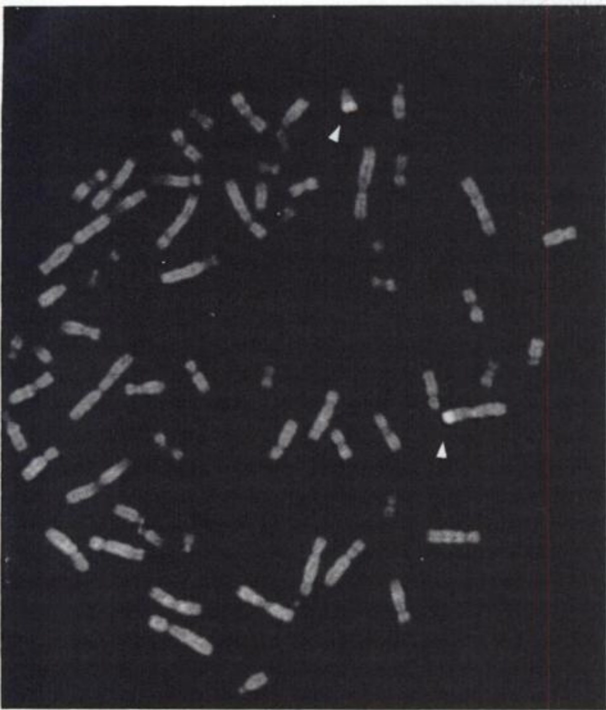

Fig. 2. A Q-banded metaphase plate. *Arrows*, normal Y with a brilliant fluorescent body and Y translocated onto no. 3.

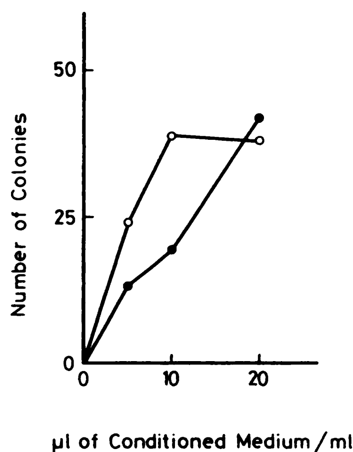

Chart 2. Dose-response relationship between number of colonies and concentrations of culture medium of T3M-1-T2 cells grown in a protein-free medium assayed against normal human nonadherent bone marrow cells. *Points*, mean colony number generated by 2×10^5 nucleated nonadherent human bone marrow cells per dish for 3 dishes. ●, without serum; ○, with 10% FBS.

protein, since protein concentration of the medium was much higher (5.35 mg/ml) in the culture.

DISCUSSION

The long-term growth of human CSF-producing cells has been established in a protein-free chemically defined medium. The cells have been propagated continuously in synthetic F-10 medium for more than 5 years. No supplements were used for the culture. The cells grew in protein-free F-10 medium with a 30-hr population-doubling time and secreted a large amount of human CSF into medium.

Intensive efforts have been made to develop human cell lines in a protein-free chemically defined medium. However, only a few human cells have been shown to proliferate in protein-free medium for a long time (4, 16). In addition, they have not expressed their differentiated functions in the protein-free cultures. Our studies have been aimed to develop human cell lines that retain their characteristic functions in a protein-free medium. Only recently, we have established a human erythroleukemic cell line in a protein-free synthetic medium which retains the characteristic function of synthesizing hemoglobin (11). Our present study shows that T3M-1-T2 cells growing in protein-free F-10 medium also retain the characteristic function of secreting human CSF (14).

On the other hand, many cell lines grow in serum-free media supplemented with several growth-supporting factors, such as insulin, transferrin, albumin, or other growth factors and hormones (1, 2). The inclusion of these factors, however, can cause confusion when we assess the biochemical changes occurring during proliferation and differentiation of the cells, since biological actions of these factors have not been elucidated fully (15), and many of them show several different biological effects on cultured cells (7, 13, 15). In addition, serum or growth factors in the medium samples may modulate the CSF activity. It has been shown that insulin, transferrin, and albumin support the colony growth of bone marrow progenitors of granulocytes and macrophages (8). When we assess the CSF activity in the conditioned medium, such factors may enhance the CSF activity. Since T3M-1-T2 cells grow and secrete CSF in a protein-free chemically defined medium, such possibilities are substantially excluded, thus facilitating the studies on regulation of CSF synthesis in human cells as well as on the mechanisms by which the cells control their proliferation.

CSF is considered to be a physiological granulopoietin which controls differentiation of precursor cells into mature granulocytes and macrophages. The studies on human CSF have been hampered by the difficulty in obtaining the material in large quantities for purification. Since multistep purification procedures usually yielded low recoveries of active materials, purification often runs into difficulties only because of too high a level of contaminating proteins in the crude materials. Although several cell lines have been reported to produce human CSF, they require serum for the proliferation (5, 6, 10, 12, 14). No cell lines have been shown to proliferate continuously or secrete CSF in a protein-free chemically defined medium. There are hundreds of different proteins with a combined total concentration of 5 to 15 mg/ml of culture fluid in medium supplemented with 10% FBS (14). Purification of human CSF from these cultures has been hindered by the complex nature and the high-protein concentration of the conditioned medium (12). T3M-1-T2 cells secreted about 2,000 units of CSF activity per ml of culture medium. The specific activity was very high (25,974 units/mg of protein), even in the crude materials, because of the low protein concentration of the medium (77 μg/ml). The use of our protein-free culture line will therefore provide a great advantage for purification of human CSF because of no contamination with serum proteins. In addition, the protein-free culture system is simple and economical for CSF purification. When we culture the cells, it is necessary to select serum suitable for the cell growth, which is very expensive and time consuming. Elimination of its use when growing CSF-producing cells on a large scale for the purpose of CSF production can result in a substantial decrease in medium cost. There is also considerable variation in the quality of different

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lots of serum. Hence, it is necessary to exhaustively test all serum lots to determine if they are suitable for the CSF-producing cells. This source variation and expense are eliminated with the use of the cells growing in a protein-free chemically defined medium.

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