Establishment and Characterization of a Human Cancer Cell Line That Produces Human Colony-stimulating Factor

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ABSTRACT

A human colony-stimulating factor (CSF) producing cell line, T3M-1, has been established from explant cultures of a human squamous cell carcinoma of the oral cavity that secretes human CSF. It has been continuously propagated during the past 15 months. The cells grew in a monolayered sheet with about 17 hr of population-doubling time and showed a colony-forming capacity with about 5% plating efficiency. The cells exhibit an epithelioid morphology resembling the structure of the original tumor, and they showed “tumor takes” when inoculated into nude mice. Karyotypic analysis revealed the cell line to be a human aneuploid one with a hypotriploid mode, including the Y-chromosome(s) and at least 10 common markers. T3M-1 cells possess the characteristic function of human CSF production in vitro, and a marked neutrophilia was observed in nude mice bearing the tumors produced by inoculation with the T3M-1 cells. In view of these characteristics, T3M-1 represents a new human cell line that secretes human CSF.

INTRODUCTION

Proliferation of granulocytes and macrophages is considered to be controlled by specific factor(s). When the specific progenitor cells of granulocytes and macrophages in the bone marrow are cultured in semisolid agar medium, they proliferate and form colonies of differentiated granulocytes and macrophages (3). This proliferation requires the presence in the medium of an adequate concentration of specific regulator(s), e.g., CSF. Although in mice many sources, including conditioned medium of L-cell cultures, are found to have CSF (2, 4, 29), the distribution of this humoral factor is more limited in humans. Several human sources including urine (30) and conditioned medium of cultures of peripheral leukocytes (9, 16), spleen (21), placenta (7, 24), embryo kidney (5), or lung (13, 28) contain CSF. However, few constant sources of human CSF have been found (10).

Without any evidence of infection, a moderate neutrophilia is sometimes observed in patients with various cancers (11, 15, 19, 25). As we reported before, such a cancer, which produced a moderate neutrophilia in a patient (OTUK tumor), was successfully transplanted into athymic nude mice (20). A marked neutrophilia was observed in nude mice bearing the tumor, and CSF was demonstrated in the tumor extract (1). Recently, we successfully transplanted into nude mice another cancer that had produced a marked neutrophilia in a 33-year-old Japanese male patient (LJC-1-JCK tumor) (26).

In an attempt to isolate a cell line from these human CSF-producing tumors grown in nude mice, the primary cultures were carried out repeatedly during the past 3 years. In the present communication we report a cell line isolated from the LJC-1-JCK tumor that has been propagated in vitro by serial subcultures in our laboratory and continuously produces human CSF.

MATERIALS AND METHODS

A 33-year-old Japanese male noticed swelling and pain of the right lower gingiva in October 1975. Under the diagnosis of squamous cell carcinoma of the oral cavity, hemimandibulectomy was performed on January 23, 1976. On July 10, 1976, he was admitted to the Tokyo University Hospital, and pleural metastases were detected. Marked neutrophilia (37,000 to 100,000/cu mm) persisted throughout his hospital course without any evidence of infection. He died of disseminated metastases of the tumor on August 21, 1976. The tumor cells obtained immediately from a pleural metastasis were transplanted into athymic nude mice (BALB/c-nu/nu mice, background congenitally athymic). The tumor has been maintained in nude mice through serial passages over a period of 1 year (LJC-1-JCK tumor). Marked neutrophilia was observed in the tumor-bearing mice, and high titers of CSF were detected in cyst fluid obtained from the tumor (26).

Such a tumor was removed from the nude mice for attempts at establishment of a CSF-producing cell line in vitro on February 24, 1977.

Athymic Nude Mice. Experiments were carried out on female congenitally athymic nude mice (BALB/c-nu/nu mice, 7 weeks old, background congenitally athymic), purchased from Clea Japan Inc. (Tokyo, Japan).

Culture Media. The medium used for the primary culture was F-10 synthetic culture medium (Flow Laboratories, Inc., Rockville, Md.) (14) supplemented with 10% FCS (Grand Island Biological Co., Grand Island, N. Y.). The medium used for serial subcultures was F-10 supplemented with 10% calf serum or FCS (Grand Island Biological Co.). Cells were harvested with 0.25% trypsin (1:250; Difco Laboratories, Detroit, Mich.) and 0.02% EDTA, in calcium- and magnesium-free balanced salt solution. Penicillin (100
units/ml) and streptomycin (100 μg/ml) were added in the medium.

**Primary Culture.** The tumor (approximately 300 mg) was rinsed twice with F-10 and minced into small pieces (1 to 2 cu mm) with scissors. The pieces were incubated in 20 ml of 0.25% trypsin solution at 37° for 20 min, and then in 20 ml of 0.02% EDTA solution at 37° for 5 min successively. The treated pieces were dispersed in 16 ml of F-10 medium containing 10% FCS, immediately dispensed into 2 Falcon 3013 flasks (25 sq cm), and incubated at 37° in a humidified atmosphere of 5% CO₂ in air.

Large epithelioid cells that migrated from the attached tissue pieces covered the bottom of the flasks. When outgrowth from explants reached confluency, the cells were harvested by the use of 0.25% trypsin and 0.02% EDTA solution successively. The cells from Flasks 1 and 2 were each dispersed into 8 flasks. One (a) was cultured in 8 ml of the same medium, the second (b) in 8 ml of F-10 containing 10% calf serum, the others (c, d, e, f) in 8 ml of F-10 containing 10% FCS of 4 different lots. After these replating procedures, the flasks were examined daily for growth, and the medium was changed every 3 to 4 days.

**Serial Subcultures.** During the subsequent 2 weeks of primary culture, it became apparent that in all flasks except Flask 1-b, a heterogeneous population of polygonal cells with big nuclei had propagated and reached confluency. Subcultures were performed on a weekly basis at 1:3 dilution in the same manner as described above. An initial rapid growth during the first 4 weeks was followed by several months of less active growth with ultimate loss of the cells. On the other hand, in Flask 1-b, a homogeneous type of polygonal cells with diffuse granules in cytoplasm had grown very slowly and reached confluency during the subsequent 6 weeks of primary culture. As few subcultures of these cells as possible were performed, and the culture was maintained only by medium changes, depending on the condition of the culture, to prevent the cells from starving. Adaptation to rapid growth on the medium was accomplished during the subsequent 5 months of continuous culture. At the time of the submission of this report, the cells had been subcultured to passage 75. During this period of continuous propagation, cells of different passages were stored in liquid nitrogen. After thawing, the stored cells could be propagated in culture without noticeable change in growth and CSF production.

**Cloning Procedure.** The original culture used for establishing a clonal strain of CSF-producing cells was derived from the subcultures of Flask 1-b. Single-cell platings, without feeder layer, were made from the culture by the method described by Puck et al. (23). Stainless steel cylinders were used for the isolation of individual colonies. The isolated colonies were detached and dispersed into single cells with trypsin and EDTA solution. The dispersed cells were counted, serially diluted, and distributed in fresh dishes. Original clonal strains were recloned twice.

**Plating Efficiency.** The cells of passage 40 were studied to estimate the plating efficiency. The initial cell number was 200 to 500 cells per dish (Falcon 3002, 60 x 15 mm). The cells were grown in the same medium as described for serial subculture.

**Transplantation into Nude Mice.** The cells (5 x 10⁶) of passage 29 were harvested in 0.2 to 0.3 ml of medium and inoculated into s.c. tissue on the back or the flank of 3 nude mice. Nodules were detected in all 3 animals after 17 days of transplantation, and the peripheral granulocyte counts were examined after 7 to 43 days of transplantation. The nude mice were sacrificed after 28 to 43 days of transplantation for histological examination.

**Growth Curve.** The cells of passage 40 were studied to estimate the population-doubling time. The initial cell number was 5 x 10⁶ cells/dish for a total of 22 dishes (Falcon, 3001, 35 x 10 mm). The cells were grown in 2 ml of medium as described for serial subculture. A cell count was taken each day in 2 dishes, and the growth medium was changed every day.

**Histological Examination.** All the extirpated tumors from the patient and from nude mice were fixed in isotonic-buffered formalin (10%, v/v), stained with hematoxylin and eosin, and examined under a light microscope. Cells in Petri dishes were photographed directly without stain by the use of a phase-contrast condenser. The primary tumors and the tumors from inoculated cultured cells and *in vitro* cultured cells were fixed with 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, postfixed in 1% OsO₄ in the same buffer, and embedded in Epon 812 after dehydration. Thin sections were cut, stained with uranyl acetate and lead citrate, and examined with an electron microscope (Hitachi HU-12; Hitachi, Ltd., Tokyo, Japan).

**Chromosomal Analysis.** For chromosomal preparations, the cultured 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 with conventional Giemsa for counting the number of chromosomes. For identification of chromosomes, the G-banding and Q-banding techniques were used (8, 27).

**Assay for CSF.** CSF was assayed 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 by Messner et al. (17), 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 x 10⁹) of nonadherent nucleated cells were 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), 15% fetal bovine serum (Flow Laboratories, Inc.) and varying concentrations of cultured medium or control medium. In control culture, cyst fluid obtained from the CSF-producing tumor (LJC-1-JCK), was used as a standard CSF (26). For studies of dose response, the original samples were diluted with McCoy's Medium 5A. After 10 days of incubation in a fully humidified atmosphere of 7% CO₂ in air, discrete colonies containing 50 or more cells were counted with an inverted microscope.
RESULTS

Morphology of a Cell Line, T3M-1, of Human Oral Cavity Carcinoma Cells. The original cell line, designated T3M-1, was established in vitro. The appearance of T3M-1 cells is shown in Fig. 1a. The cells were characterized by an epithelial-like shape with prominent nuclei. Nuclei were generally found localized at the end of the cytoplasm and were pleomorphic in shape, usually containing 2 to 6 prominent nucleoli. The cell membranes were usually rough, sometimes with extending appendage(s). The cells formed characteristic colonies which coalesced and formed a monolayer. Electron micrographs revealed that most individual cells were characterized by prominent nuclei with multiple large nucleoli. The cells had numerous microvilli at the plasma membrane. The cytoplasm revealed elongated mitochondria and Golgi complexes. The granular endoplasmic reticulum and numerous free polysomes were also observed. Bundles of tonofilaments were found most commonly associated with desmosomes and in the regions adjacent to the apical membrane (Fig. 2, a and b). No virus-like particles were observed in any of the cells examined. These features suggested that the cells were epithelial in origin, especially derived from a differentiated squamous cell carcinoma. This ultramicrostructure was similar to that of the original tumor maintained in nude mice.

CSF Production in T3M-1 Cell Culture. Confluent culture of T3M-1 was incubated with complete growth medium. One week after the incubation, medium was harvested and dialyzed against distilled water for 3 days. CSF was assayed by the use of normal human nonadherent bone marrow cells as target. The undialyzed cultured medium failed to reveal activity; dialysis was necessary for the cultured medium to develop activity. Representative results are shown in Chart 1. Cultured medium of T3M-1 stimulated colony formation in a dose-dependent manner. No colony formation was stimulated by the addition of control medium (F-10 + FCS).

The titers of CSF produced in vitro by serial subcultures of T3M-1 are listed in Table 1. One year after the primary culture, T3M-1 cells of passage 57 could be propagated in culture without noticeable change in CSF production. A clonal strain of the cell line was derived from the subculture of Flask 1-b. The cultured medium of these clonal cells also had a higher CSF titer.

Growth Characteristics. A kinetic study on the in vitro growth of the cultured T3M-1 cells is shown in Chart 2. The cells used for these experiments were the 40th-transfer generation cells, completely adapted to the in vitro condition and growing vigorously.

The experimental cultures were initiated with an inoculum of 5 x 10⁴ cells and incubated in a CO₂ 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 2, the population-doubling time was estimated as 17 hr. The plating efficiency of the T3M-1 cells was about 5%.

Growth in Nude Mice. The growth of the cultured cells,

Table 1

<table>
<thead>
<tr>
<th>Passage</th>
<th>Date of examination</th>
<th>CSF activity at concentrations of Standard CSF²</th>
<th>(10%)</th>
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<tr>
<td>13</td>
<td>8/21/77</td>
<td>0%</td>
<td>68.0</td>
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<tr>
<td>24</td>
<td>9/28/77</td>
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<tr>
<td>Clonal²</td>
<td>10/22/77</td>
<td>0%</td>
<td>56.0</td>
</tr>
</tbody>
</table>

² Mean colony number formed by 2 x 10⁴ nucleated nonadherent bone marrow cells per dish for 3 dishes tested.

² Inasmuch as data from 4 separate experiments are shown in this table, the number of colonies stimulated in each experiment by a standard CSF is given for reference.

A clonal strain of the cell line was derived from the subculture in Flask 1-b and subcultured to passage 18.

Chart 1. Dose-response relationship between number of colonies and concentrations of cultured medium of T3M-1 cells assayed against normal human nonadherent bone marrow cells. Each point represents the mean colony number generated by 2 x 10⁴ nucleated nonadherent human bone marrow cells per dish for 3 dishes.

Chart 2. Growth curve of cultured cells at the 40th transfer generation of T3M-1 under 5% CO₂ atmosphere. Each point represents the mean value from 2 dishes.
Human CSF-producing Cell Line in Vitro

T3M-1, was tested to see whether the cells were capable of growing in nude mice and what type of tumor would develop. The results of the retransplantation out of tissue culture showed that all cases were successful, the animals showing perfect tumor takes.

All the tumors became palpable by 8 to 17 days and grew to a size of 300 to 4000 cu mm by 2 to 3 weeks. Nude mice bearing the tumors from the inoculated T3M-1 cells also developed a marked neutrophilia in parallel with the tumor growth (Chart 3).

Fig. 1b shows typical features of a tumor produced in a nude mouse. The histology of the solid tumor obtained by inoculating the cultured T3M-1 cells into nude mice was similar to that of the original tumor grown in nude mice after direct transplantation from a pleural metastasis of the human tumor. Both tumors were relatively well-differentiated squamous cell carcinomas with occasional pearl formation. The original tumor frequently formed cystic cavities lined by squamous cells, and a small number of neutrophils were scattered within the tumor (Fig. 1c). The tumor produced by inoculating the cultured cells showed rather more prominent keratinization than the original one. Electron micrographs revealed that most of the cells had bundles of tonofilaments, mitochondria, Golgi complexes, glycogen areas, granular endoplasmic reticulum, and numerous polysomes. The adjacent cells were united with the desmosomal junctions. The cells were covered with numerous microvilli (Fig. 2c). The results of observations suggested that the cultured cells were histologically similar to the original tumor cells.

Chromosomal Findings. The modal chromosome number of the T3M-1 cell line was 66 in the 13th and the 36th passages, and 68 in the 48th passage, with considerable scattering in counts (Table 2). Not a single normal metaphase was observed. The Q-bandings and G-bandings techniques revealed the presence of at least 10 common markers, although several other uncommon markers were present. Of all the marker chromosomes, 10 common ones that were fully or partially identifiable are shown (Fig. 3a).

M1 was a large subacrocentric chromosome of the size of Chromosome 3. Most of the long arm of the marker is similar to the long arm of No. 2 (break at band 2q11).

M2 was a metacentric chromosome of the size of No. 3. The marker probably originated by translocation of Yq onto the short arm of No. 3 (break at band 3q21-23). This structure was more distinct with Q-bandings than with G-bandings, because of a brilliant fluorescent body as distal Yq on the marker. A normal Y-chromosome was present besides the translocated Y in all of the metaphases observed (Y in Fig. 3, a and b).

In M3, the long arm was homologous to that of No. 4. The short arm was unknown.

M4 probably has arisen by translocation of the distal half of the long arm of No. 1 (break at band 1q21–23), probably onto the long arm of No. 8 (break at band 8q22).

M5 was slightly shorter than M4. The marker originated due to deletion in 1q; i.e., del(1)(q21–22).

The size of M6, a metacentric marker, was nearly equal to No. 5. One arm was apparently homologous to No. 14.

One arm of M7, a metacentric marker, was similar to the long arm of No. 10.

M8 was a metacentric chromosome of the size of the D-group chromosomes. It probably involved a translocation between 18q and 17q.

M9 was a metacentric chromosome equal in size to MN. Identification of this marker was rather difficult, because of several possibilities. Both of the arms were similar to the short arms of No. 4, No. 5, and No. 11.

M10 probably originated by deletion of the short arm of No. 16; i.e., del(16)(p12).

DISCUSSION

This study describes a newly established cell line derived from a human CSF-producing squamous cell carcinoma from a Japanese male patient. The original cell line, designated T3M-1, has been propagated continuously by serial subcultures during the past 15 months and produces human CSF continuously in vitro.

A major limitation to the usefulness of cell cultures is the general inability to prevent alteration in cellular properties when cells are transferred from their environment in vivo to the condition of culture in vitro (6, 12).

The tumors producing bioactive substances have been known as "functional tumors." Instability of such functional properties in the cultured cells in vitro is quite possibly a result of the vigorous growth of the cells (22, 31, 32).
Selectove overgrowth of the cultured cells from functioning tumors may be in favor of establishing nonfunctioning cell lines, but it threatens the functional status of cultures. In order to prevent selective overgrowth and to obtain CSF-producing cell cultures, we used the calf serum that might be worse for the cell growth in the early period of the culture. The demonstration of CSF production by T3M-1 cells supports the view that the CSF production characteristic of the original tumor cells has been maintained during the course of cellular adaptation to an in vitro environment. This study emphasizes the remarkable stability of functional properties as well as morphological features possible in cell cultures and in heterotransplanted tumors.

Without any source of large amounts of human CSF, we could not produce any distinct evidence showing that granulocytosis might be produced in humans and animals when they were injected with CSF, despite the apparent in vitro effects of CSF on granulocyte proliferation. However, CSF is still considered to be an important regulator of availability of the CSF-producing cell line, T3M-1, makes the inoculated T3M-1 cells. Human CSF-producing cell could not produce any distinct evidence showing that marked neutrophilia in nude mice bearing the tumors from cultures and in heterotransplanted tumors.

The demonstration of CSF production by T3M-1 culture supports the view that the CSF production character of the original tumor cells has been maintained during the order to prevent selective overgrowth and to obtain CSF-tumors may be in favor of establishing nonfunctioning cell lines, but it threatens the functional status of cultures. In order to prevent selective overgrowth and to obtain CSF-producing cell cultures, we used the calf serum that might be worse for the cell growth in the early period of the culture. The demonstration of CSF production by T3M-1 cells supports the view that the CSF production characteristic of the original tumor cells has been maintained during the course of cellular adaptation to an in vitro environment. This study emphasizes the remarkable stability of functional properties as well as morphological features possible in cell cultures and in heterotransplanted tumors.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. Morphological features of the cultured T3M-1 cells, the original tumor grown in nude mice, and the tumor produced by inoculation with T3M-1 cells. 

a. monolayer-cultured T3M-1 cells were epithelial-like in shape (the 44th transfer generation). Phase-contrast microscopy, $\times$ 500. 

b. the histology of the tumor obtained by inoculating the 29th transfer generation of T3M-1 cells into nude mice has the appearance of a relatively well-differentiated squamous cell carcinoma. H & E, $\times$ 300. 

c. the histology of the original tumor grown in nude mice after direct transplantation from a pleural metastasis of the tumor, showing a relatively well-differentiated squamous cell carcinoma. H & E, $\times$ 300.
Fig. 2. Electron micrographs of the monolayer-cultured T3M-1 cells and the tumor obtained by inoculation with T3M-1 cells. a, electron micrograph of the T3M-1 cells (the 40th transfer generation) revealed bundles of tonofilaments (T), elongated mitochondria (M), numerous polysomes, and granular endoplasmic reticulum (ER). × 27,000. b, electron micrograph of the T3M-1 cells (the 40th transfer generation), showing bundles of tonofilament (T) associated with desmosomes (D). × 42,000. c, electron micrograph of the tumor obtained by inoculating the 29th transfer generation T3M-1 cells into nude mice revealed bundles of tonofilaments (T), desmosomes (D), Golgi complex (G), granular endoplasmic reticulum (ER), glycogen area (Gly), and numerous polysomes. × 32,000.
Fig. 3. Chromosomal findings of the T3M-1 cell line. a, partial karyotype consists of 10 G-banded markers, 1 Q-banded M₅ marker and a normal Y-chromosome with a brilliant fluorescent body, seen commonly in the T3M-1 cells (the 46th transfer generation). b, a Q-banded metaphase plate. Arrows, normal Y with a brilliant fluorescent body and a Y translocated onto No. 3.
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