Development of a 3T3-like Line from an Embryo Culture of an Inbred Strain of Syrian Golden Hamster

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SUMMARY

An embryo culture of an inbred strain of Syrian hamster developed into a permanent cell line under the "3T3" culture regimen, i.e., transfer every 3 days of an inoculum of 3 \times 10^6 cells/60-mm dish. The resulting cell line had properties very similar to those of the mouse 3T3 series and was named HAMS 3T3. The cells showed density-dependent inhibition of division with a saturation density of 1.0 to 1.2 \times 10^6 cells/60-mm dish or 4.5 to 5.5 \times 10^4 cells/sq cm. Addition of fresh medium containing 5 or 10% fetal calf serum to a confluent culture induced DNA synthesis in 18 hr with subsequent cell division. Cells were hyperdiploid with a mode of 45 chromosomes (80% of the cells). When cells at the 60th passage were injected into the skin or cheek pouch of an inbred hamster of the same strain as that from which they were derived, they produced a benign tumor that regressed after 3 weeks. Morphological transformation was obtained by infection with the Moloney strain of murine sarcoma virus.

INTRODUCTION

Established mouse cell lines of the 3T3 series have been used widely for a variety of studies on control of cell division and transformation by oncogenic viruses and chemicals, because they show "density-dependent inhibition of division," a term proposed by Stoker and Rubin (30). Todaro and Green (31) and Aaronson and Todaro (1) established these 3T3 cells from a random-bred Swiss mouse embryo and from an inbred BALB/c mouse embryo, respectively, using a culture regimen that minimizes cell contact. The term "3T3" designates this subculturing regimen, i.e., transfer at 3-day intervals of a relatively low inoculum of 3 \times 10^6 cells/60-mm Petri dish. Cell lines with properties very similar to those of the 3T3 series have been established from kidney (37), an organ culture of prostate (5, 24), and embryos (27) of C3H mice, and from rat embryos (17). Todaro et al. (34) reported that under the 3T3 transfer schedule, hamster embryo fibroblast cultures always grew well initially but failed to develop into established lines, while polyoma virus-induced tumors always gave rise to established lines. This study was of the establishment and properties of a 3T3-like cell line from an embryo culture of an inbred strain of Syrian hamster.

MATERIALS AND METHODS

Cell Culture. Eagle's minimum essential medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10% FCS (Grand Island Biological Co., Grand Island, N. Y.) was used. The cells were maintained in 60-mm plastic Petri dishes (Falcon Plastics, Oxnard, Calif.) and were subcultured using 0.1% trypsin (Difco Laboratories, Inc., Detroit, Mich.) in PBS. Cultures were incubated in a humidified incubator at 37°C under an atmosphere of 5% CO2 in air.

The primary culture was initiated by fine mincing and trypsin (0.25% in PBS) digestion of a near-term whole embryo of an APG Syrian hamster. This strain was established by brother-to-sister mating for more than 50 generations in the Laboratory of Experimental Animals of this institute. The primary culture originating from 1 embryo was dispensed into 3 separate cultures (H-1 to H-3). These cultures were then put on a rigid transfer schedule, following the method of Todaro and Green (31), with transfer every 3 days using an inoculum of 3 \times 10^5 cells/60-mm Petri dish. The medium was not changed during the 3-day subculture periods. At the stage of reduced growth, the cultures often did not yield enough cells to allow transfer of 3 \times 10^5 cells/dish by the 3rd day, so either 3 \times 10^5 cells were obtained by pooling cells from 2 dishes or a lower inoculum was used. Cumulative growth curves, representing the cumulative increase in cell number against the number of days in culture, were plotted based on the cell proliferation in each transfer, taking the inoculum size of the 1st subculture as 1.

Plating efficiency was measured at various passage levels by plating 100 to 1000 cells, depending on their plating efficiency, in 60-mm dishes. Agar suspension culture was done following the method of MacPherson and Montagnier (22). The procedure used for agar plate culture has been described elsewhere (18, 19).

Chromosome Preparations. Exponentially growing cells were arrested in metaphase by treatment with approxi-
mately $5 \times 10^{-7}$ M colchicine for 2 hr. The cells were then treated by the procedure of Yosida et al. (38). Preparations were stained with Giemsa.

**Transplantation.** The tumorigenicity of the cells was examined in APG hamsters, bred in our laboratory by brother-to-sister mating. Adult animals were inoculated with $2 \times 10^5$ cells in 0.1 ml of complete medium by injection into the skin or cheek pouch. Animals were not conditioned before inoculation by irradiation or cortisone treatment and were observed for at least 4 months after the inoculation.

**Induction of DNA Synthesis.** Samples of cell suspension containing $7 \times 10^5$ cells in 0.5 ml were mounted on 25-mm round plastic coverslips (Lux Scientific Co., Thousand Oaks, Calif.) and placed in 35-mm Petri dishes. This cell density corresponded to an inoculum of $3 \times 10^5$ cells/60-mm dish or $1.4 \times 10^4$/sq cm. After incubation overnight, 1 ml of medium was added in each Petri dish. On the 5th day when the cells had stopped growing, the medium was replaced by fresh medium and DNA synthesis and cell growth were measured at 1- or 2-hr intervals.

To measure DNA synthesis, cells were pulse-treated with $[\text{methyl-}^3\text{H}]$thymidine (13 Ci/m mole; Daiichi Chemical Co., Tokyo, Japan) at a concentration of 0.5 µCi/ml for 15 min. Then the cells were washed twice with PBS, fixed with methanol, and treated with 0.5 N perchloric acid for 20 min at 4°C. Coverslips were placed in vials containing 10 ml toluene scintillator and 0.5 ml Soluene-100 (Packard Instrument Co., Downers Grove, Ill.) and counted in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). For autoradiographs, coverslips were coated with Sakura NR-M1 emulsion (Konishiroku Photo Co., Ltd., Tokyo, Japan). After exposure for 1 week, autoradiographs were developed in Koplinal (Fuji Photo Film Co., Ltd., Tokyo, Japan) for 5 min at 20°C and stained with Giemsa. Over 500 cells were examined to determine the percentages of cells synthesizing DNA and in mitosis.

**Transformation Experiments.** Transformability was investigated using MSV and various chemicals. For transformation with MSV, cultures in the logarithmic growth phase, containing $1 \times 10^4$ cells/60-mm dish, were pretreated with 25 µg DEAE-dextran per ml (Pharmacia Fine Chemicals AB, Uppsala, Sweden) for 1 hr and then washed and treated with 0.5 ml of 10-fold dilutions of the Moloney strain of MSV for 1 hr. Virus stock containing $10^6$ focus-forming units/ml was prepared from the culture fluid of a clonal line of MSV-transformed rat brain tumor (C2-RBT), obtained from Dr. N. Ida, Toyo Kogyo Hospital, Hiroshima, Japan (15). Morphological transformation was observed for a period of 3 weeks. The medium containing 2% FCS was changed twice weekly. Culture fluid was tested for the presence of MSV by assay of focus formation with YH-7 cells (36) and for MLV by the XC test (28).

In attempts to induce transformation by chemical carcinogens, DMBA and MNNG were used as follows. Cells, $10^4$ were seeded per 60-mm dish and on the next day the test chemicals dissolved in DMSO were added at a final concentration of 0.5% DMSO. Control cultures were treated with 0.5% DMSO only. The duration of exposure was 48 hr. The medium containing 10% FCS was changed twice weekly. After 6 weeks, the dishes were fixed and stained with Giemsa.

**RESULTS**

**Establishment of the HAMS 3T3 Cell Line.** Chart 1 indicates the cumulative growth curves of 3 cultures (H-1 to H-3) of hamster fibroblasts, initiated from a primary culture of a single embryo as described in the “Materials and Methods.” When transferred successively every 3 days at an inoculum of $3 \times 10^6$ cells/60-mm dish, the cells grew actively during the 1st 30 days with an approximate doubling time of 35 hr, judged from their cumulative growth curves. However, when the cumulative increase in cell number of the culture H-1 and H-2 reached approximately 50

![Chart 1. Cumulative growth curves of 3 embryo cultures of the inbred Syrian hamster, all derived from a primary culture of single embryo and transferred by the “3T3” regimen, i.e., transfer every 3 days of an inoculum of $3 \times 10^6$ cells/60-mm dish. Three phases of growth were seen. First (0 to 30 days), cells grew actively with a doubling time of 35 hr, then (30 to 70 days) growth decreased, and then cells showed stable, steady growth. Cultures H-2 and H-3 died in the 2nd phase, while H-1 overcame the critical stage and developed into a permanent cell line, HAMS 3T3. Ordinate, cumulative increase in cell number, taking the cell number at the 1st subculture as 1.](image-url)
10^4 to 10^5 after about 30 days of culture, their growth rates decreased and their doubling times after 30 to 65 days were 100 and 120 hr, respectively. The H-3 culture showed a plateau at 10^4 in the cumulative growth curve for more than 60 days. This period seemed to correspond to the decreased growth rate observed after 45 to 75 days during establishment of mouse 3T3 cells (1,31) and also to Phase III in aging of human diploid fibroblasts (12). Cultures H-2 and H-3 gradually lost their ability to divide in culture and eventually died on Days 88 and 104, respectively, at cell numbers of 10^40 and 10^12 in their cumulative growth establishment of mouse 3T3 cells (1,31) and also to Phase III in aging of human diploid fibroblasts (12). Cultures H-2 and H-3 gradually lost their ability to divide in culture and eventually died on Days 88 and 104, respectively, at cell numbers of 10^40 and 10^12 in their cumulative growth curves. Culture H-1 overcame this critical stage and began to divide actively after 70 days in culture, showing a doubling time similar to that at the beginning of culture. This line has now been cultured for over 100 transfers. It has a constant growth rate and has never shown any indication of dying out so that it may now be regarded as established; it is named "HAMS 3T3."

Growth Properties of HAMS 3T3 Cells. During the 1st 2 days the cells proliferated with a doubling time of 19.4 hr and then reached a saturation density, which was usually 1.0 to 1.2 x 10^4 cells/60-mm dish (4.5 to 5.5 x 10^4/sq cm). When cultured at confluence for more than 3 days without a change of the medium, the cells began to degenerate and become detached. However, when the medium was renewed the cells quickly recovered and grew to confluence. This experiment indicates that HAMS 3T3 cells, like mouse 3T3 cells, are sensitive to density-dependent inhibition of division and also to serum deprivation.

Morphologically, HAMS 3T3 cells were fusiform with the typical appearance of fibroblasts. In confluent culture the cells displayed a parallel orientation with no tendency to form multilayers (Fig. 1). This again indicates that HAMS 3T3 cells are sensitive to density-dependent inhibition of division.

The plating efficiency of this cell line was investigated periodically during the course of its establishment. The plating efficiency increased with time of cultivation. The efficiencies at the 23rd and 39th passage were 1.2 and 4.4%, respectively, while at the 55th passage almost 70% of the cells plated formed colonies. This is in good agreement with the report of Todaro and Green (31), that during establishment of the Swiss 3T3 line the cells gradually acquired the ability to proliferate from small inocula. So far, HAMS 3T3 cells have failed to form colonies either in agar suspension culture or on agar plates.

Chromosome Constitution. In most cases during establishment of cell lines the chromosomal constitution changes. This cell line was also accompanied by a shift to a hyperdiploid constitution with a mode of 45 chromosomes. In the early stage (at the 27th passage) 22.2% of the cells had 45 chromosomes and 14.8% were diploid, while at the 60th passage 81.5% of the cells had 45 chromosomes.

The karyotypes of HAMS 3T3 are shown in Fig. 3, where the chromosomes are classified and arranged according to shape and size, as reported previously (38). All cells had 2 X chromosomes (the largest being submetacentric), indicating that the cells originated from a female embryo. Karyograms of the cells with 45 chromosomes were characterized by elimination of 1 or 2 chromosomes of the A group (large, subtelocentric) and 2 trisomies or tetrasyomies of the E group (small subtelo- or submetacentric).

Transplantation of Cells. The tumorigenicity of the HAMS 3T3 line was examined by inoculating the cells into the same inbred strain of hamster as that from which they were derived. After inoculation of 2 x 10^4 cells at the 60th passage either s.c. (6 animals) or into the cheek pouches (3 animals) of adult hamsters, small nodules (approximately 5 x 5 mm) were found in all animals in 1 to 2 weeks, but later these nodules regressed. Histologically, necrosis was seen at the center of tumors surrounded by sparsely proliferated fibroblastic cells. No tendency for infiltrative proliferation into the muscle layer was seen. The tumors were diagnosed histologically as benign fibromas.

Hard, white nodules were found at the site of inoculation into the cheek pouch 4 months after inoculation. Histologically, the nodules consisted of bone cells and a collagen matrix depositing calcium. It is uncertain whether the HAMS 3T3 cells were converted to bone cells in the cheek pouch or whether bone tissue was induced by the transplantation.

Induction of DNA Synthesis and Cell Division by Change of Medium. It has been found with 3T3 cells and other similar cells that addition of fresh medium to a confluent culture, in which most cells are not synthesizing DNA, induces DNA synthesis and cell division (13, 33, 37). This was also the case with HAMS 3T3 cells, as shown in Chart 2. The medium containing 10% FCS was changed on the 5th day after plating, when the cells had become confluent. DNA synthesis began to increase about 10 hr after renewal of the medium and was maximal after 18 hr, as judged either by incorporation of radioactivity (Chart 2, top) or by the labeling index (Chart 2, center). At the time of maximal synthesis, nearly 60% of the cells were in the S phase. DNA synthesis was followed by cell division (Chart 2, bottom): the peak of mitosis was observed 22 hr after the medium was changed and the number of cells had increased 2-fold after 24 hr. At the time of the peak of mitosis, about 7% of the cells were in the M phase.

Induction of DNA synthesis was found to depend on the concentration of serum in the medium. FCS concentrations of more than 5% induced DNA synthesis, while with 1 or 2% FCS no peak of DNA synthesis was found within 24 hr after renewal of the medium. Therefore, the factor inducing cell replication seems to be present in FCS and may be the same as the "serum factor" required by mouse 3T3 cells (13, 33).

Transformation Experiments. The properties of HAMS 3T3 cells described above show that these cells are suitable for analysis of in vitro transformation by oncogenic viruses and chemicals.

There are several papers on transformation of hamster cells by MSV; Zavada and MacPherson (39) reported that the Harvey and Moloney strains of MSV caused transformation of hamster embryo cells as well as BHK-21 and Nil-2 cells, which are cell lines of hamster. It was demonstrated (3, 10, 14) that transplantable hamster tumors induced by MSV did not produce infectious virus but were rescued by superinfection with MLV.

Exponentially growing cells were infected with the Moloney strain of MSV, as described in the "Materials and
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Chart 2. Induction of DNA synthesis and cell division by renewing the medium on a confluent culture of HAMS 3T3. Cells at the 66th passage were cultured on round plastic coverslips. On the 5th day, when the cells had stopped growing, the medium was replaced by fresh medium containing 10% KCS and DNA synthesis and cell division were estimated at 1- or 2-hr intervals. Top, incorporation of [3H]TdR into the acid-insoluble fraction after pulse-labeling for 15 min; center, percentage of cells labeled with [3H]TdR; bottom, number of cells/dish (d) and mitotic index. The medium was changed at zero time.

Methods. A change in morphology was first noticed 4 days after infection. The cells in the infected cultures were spindle shaped and refractile, and they grew in a disordered criss-cross fashion forming a multilayer (Fig. 2), while control cells formed a confluent monolayer without cell overlap (Fig. 1). This clearly indicates that the Moloney strain of MSV caused morphological transformation of the HAMS 3T3 cells in vitro. The transformed cells were distributed evenly over the Petri dish and showed little tendency to form distinctive transformed foci, even in dishes infected with diluted virus stocks. It was found that the culture fluid of the transformed cells produced typical transformed foci at $3.6 \times 10^8$ focus-forming units/ml on YH-7 cells and also produced MLV at $1.4 \times 10^4$ plaque-forming units/ml when examined by the XC test.

The primary object of this work was to obtain a cell line of Syrian hamster suitable for study of transformation with chemical carcinogens, since hamster embryo cells have been used preferably for such studies (4, 20, 21). Some cell lines, such as BALB 3T3, 10T1/2, and mouse prostate, were reported to be transformed by chemical carcinogens (6, 8, 16, 24, 26). Chemical transformation of the HAMS 3T3 cells was attempted using DMBA and MNNG. Cells at the 40th passage and their clones were treated with DMBA at concentrations of 0.5 and 2.0 µg/ml and with MNNG at concentrations of 0.2 and 1.0 µg/ml, respectively, for 48 hr as described in the "Materials and Methods." However, any sign of transformation, distinguished by lack of density-dependent inhibition of division, was not observed within 6 weeks after treatment.

DISCUSSION

For some unknown reason normal cells in culture develop into a permanently growing cell line in the absence of any causative agents. In most cases this change is associated with a shift from a diploid constitution. Cells of different species behave in characteristic ways with regard to their life-span as diploid cells and their frequency of development into a cell line. After the phase of reduced growth, mouse embryo cells have a very high probability of developing into permanent cell lines (1, 2, 4, 9, 31). Several investigators have reported that embryo cultures of Syrian hamster hardly grow beyond a limited number of days (4, 20, 34), whereas there is a number of cell lines of Syrian hamster (7, 11, 23, 29, 35). It was found by Todaro and Green (32) and Matsuya and Yamane (25) that raising the protein level of the medium by adding crystalline bovine serum albumin, 20 mg/ml, eliminated the phase of reduced growth of hamster cells and the cells eventually underwent a spontaneous transformation, but the role of albumin was not known. In previous studies (21) on transformation of hamster embryo cells by treatments with chemical carcinogens, 2 of 12 untreated control cultures transformed spontaneously after about 260 days of culture. In this study a permanent cell line was established from 1 of 3 independent cultures, all derived from a primary culture of a single embryo and cultured using an identical transfer schedule. Thus it seems likely that hamster embryo cells develop into a permanent cell line by chance with an incidence of roughly 10 to 30%, varying according to the culture conditions.

The characteristics of the established cell lines largely depended on the culture regimen used for their establishment. Cells established with a sparse cell transfer system, such as transfer at 3-day intervals of $3 \times 10^4$ cells per 60-mm dish, showed density-dependent inhibition of division (1, 31); cells established without special precautions about the subculture regimen usually showed piled-up growth with a criss-cross arrangement of cells (21). Recently, Reznikoff et al. (26) reported the establishment of a line, 10T1/2, from embryos of C3H mice obtained by transfer every 10 days of $0.5 \times 10^5$ cells/60-mm dish. These cells showed density-dependent inhibition of division. More recently, as reported by Kimura et al. (17), a similar cell line of rat embryo, 3Y1, was established by transfer every 3 days of $1.0 \times 10^5$ cells/60-mm dish. We established a similar cell line from a Syrian hamster embryo by transfer every 6 days of $3 \times 10^4$ cells/60-mm dish (6T3). Its characteristics are now under investigation.
ACKNOWLEDGMENTS

The authors wish to thank K. Katayanagi for skillful assistance and Dr. K. Suzuki of this Institute for kindly supplying the APG strain of Syrian hamster cells.

REFERENCES

Fig. 1. Phase contrast picture of confluent HAMS 3T3 cells at the 100th passage. Note the parallel orientation of cells with no indication of multilayers. 10 × 5.

Fig. 2. Transformation of HAMS 3T3 cells by infection with the Moloney strain of MSV. The cells are spindle shaped and grow in a disordered criss-cross fashion forming a multilayer, in contrast to cells in uninfected control cultures shown in Fig. 1. 10 × 5.

Fig. 3. Karyograms of HAMS 3T3 cells at the 27th passage (top) and 60th passage (bottom). Note trisomy and tetrasomy of the E group chromosome and elimination of chromosomes from the A group (at the 60th passage).
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