Establishment in Vitro of a Human Neurogenic Sarcoma


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SUMMARY

A human neurogenic sarcoma was established in vitro and maintained in continuous growth for over 5 years. The cells exhibit morphological characteristics of neurosarcoma at the light and electron microscope level. The karyotype is aneuploid (near-triploid to near-tetraploid) and presents marker chromosomes. The doubling time is 63 hr. The generation time is 58 hr, and the length of the different phases of the cell cycle are G1, 27.5 hr, S, 22 hr, and G2, 8.5 hr. An estimated 90% of the cells are in the proliferative pool. This cell line, designated T2, is suggested as a working in vitro model for human neurosarcoma. Preliminary data with the use of immunofluorescent techniques indicate that the cells may possess a common sarcoma tumor antigen.

INTRODUCTION

There has recently been a renewed interest in the establishment of permanent cell cultures from human sarcomas. The reason for this upsurge of interest lies in the possibility of utilizing these cells for basic cancer research. The field of tumor immunology has specifically benefited from these studies as shown by the observation that specific tumor antigens, probably viral dependent, could be demonstrated in some of the cell lines obtained from human osteogenic sarcomas (8, 10). Other potential uses for these cultures lie in the area of cell kinetics and chemotherapy studies in vitro (2, 7).

Until the present, we were not aware of any cell line obtained from a human neurogenic sarcoma. We are reporting the establishment of a long-term culture from an explant obtained from a neurofibrosarcoma in the right leg of a 42-year-old woman. This cell line has been growing in our laboratory for over 5 years and has been characterized by a series of morphological and kinetic parameters which included light microscopy and cytogenetic, ultrastructural, and cell kinetics investigation.

MATERIALS AND METHODS

Tissue Culture. A fragment of the original tumor obtained from the soft tissues of the upper end of the right tibial region was minced under sterile conditions, and small explants were placed in 2 T-30 flasks under a perforated cellophane membrane as previously described (11). F-10 medium, supplemented by 20% fetal calf serum, glutamine, vitamins, and antibiotics, was the nutrient mixture. The medium was partially changed every 3 days; sufficient conditioned medium was allowed to remain in the flask. The explants were periodically examined under an inverted microscope for evidence of growth. Serial passage was accomplished by previously described methods (4). After 7 months of continuous growth, the line was considered established, and several aliquots were frozen for further examination. The stock culture has been maintained in continuous culture for over 5 years.

Morphological Studies. A portion of the original biopsy was subjected to routine histopathological techniques for diagnosis. The actively growing cultures were seeded in 100-mm Petri dishes containing a sterile slide and were allowed to grow for 1 week. The slides were rinsed in 0.9% NaCl solution, air dried, and submitted to special stainings with hematoxylin and eosin, periodic acid-Schiff, Sudan black, Oil red, colloidal iron, Mayer’s, Wright’s, Alcian blue, methyl green-pyronin, and Landing’s stain for lipids (1).

Ultrastructural Studies. Samples of the original tumor were immediately fixed in 3% glutaraldehyde, postfixed in osmium tetroxide, dehydrated, and embedded in Epon. Sections 1 µm thick were cut and stained with methylene blue for orientation and selection of representative areas. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a Hitachi 11A electron microscope. Ultrastructural investigation of the tissue culture material was done on pellets obtained from the harvest of a single culture and centrifugation of the cell suspension. The pellet was fixed in 2.5% glutaraldehyde at pH 7.2 for 5 min and postfixed in 1% osmium tetroxide for 1 hr. The cells were embedded in Epon, and ultrathin sections were stained with 1% uranyl acetate and lead citrate and examined in a Siemens-Elmiskop IA electron microscope.

Cytogenetic Studies. Chromosomal analyses were initiated 4 months after the initial explant and were repeated at 4-month intervals thereafter. To obtain a large number of metaphases, we selected a 2-day culture that showed many rounded, mitotic cells. Colcemid (0.02 µg/ml) was added and the cells were incubated for 6 hr. The cells were then trypsinized and washed in 0.9% NaCl solution, and hypotonic treatment was carried out with a 1:3 dilution of the medium in distilled water. The cells were fixed with 50% acetic acid for 30 min and stained with 2% acetoorcein. "Squash" preparations were made, at least 15 well-spread intact metaphases were

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photographed, and the total number of chromosomes per metaphase was recorded. Enlargements of the pictures were utilized for the construction of a karyotype. Chromosomes were cut out and arranged according to the Denver-Chicago classification.

**Kinetic Studies.** For a determination of the doubling time, $5 \times 10^5$ cells were seeded into 60-mm Petri dishes and allowed to reach logarithmic growth. Every 12 hr, 2 replicates were harvested, and aliquots were counted in triplicate with an electronic particle counter (Coulter Electronics, Hialeah, Fla.). The values obtained were analyzed by statistical methods, with linear regression techniques and with the assistance of a 7094 IBM digital computer.

The mitotic index was determined in logarithmically growing cells. After harvest, the cells were submitted to hypotonic treatment with 0.6% sodium citrate and were fixed in Carnoy's solution. Slides were prepared by the flame-dry method, and at least 2000 cells were counted by 2 independent observers.

We carried out cell cycle analysis by seeding $3 \times 10^5$ cells in replicate Petri dishes and allowing the cells to reach logarithmic growth. They were then pulse labeled with tritiated thymidine (1 $\mu$Ci/ml; specific activity, 3.0 Ci/mmole) for 30 min. Following a double wash with fresh medium, the cells were further incubated with medium containing thymidine, 10 $\mu$g/ml. Samples were harvested every 2 hr for 120 hr. Following hypotonization and fixation, the cells were flame dried on slides. Radioautography was achieved by dipping the slides in a 50% solution of Ilford K5 emulsion and exposing the cells for 1 week. At least 100 metaphases per sample point were classified as labeled or unlabeled.

Continuous labeling was carried out by dispensing tritiated thymidine (2 $\mu$Ci/ml; specific activity, 3.0 Ci/mmole) to replicate dishes. Harvesting and radioautography were performed as described above.

**RESULTS**

The routine histological section presented a tumor composed of sheets of bizarre cells with large nuclei, coarse chromatin with a prominent nucleolus, and pale cytoplasm (Fig. 1). Some cells exhibited cytoplasmic appendages in a dendrite-like fashion. At the electron microscope level, the cells presented a loose arrangement; the nuclei were large and irregular and displayed multiple indentations. The chromatin was coarsely clumped, and several nucleoli were frequently noted. The cytoplasm appeared very dense and contained variable amounts of rough and smooth endoplasmic reticulum. Most of the cells contained numerous bundles of microfibrils and microtubules running parallel along the longitudinal axis. The cell membrane was very irregular with many processes and interdigitations, enveloping parts of neighboring cells in a fashion similar to Schwann cells (Fig. 2). In addition, many of the cells had membrane specialization areas, desmosome-like bodies (6).

The cultures were started in April 1966. A few explants demonstrated peripheral growth. From the beginning, the cells were large, polygonal, and occasionally multinucleated (Fig. 3). Four weeks later, the 1st passage was carried out, and the cells were henceforth cultured serially every 1 or 2 weeks. In November 1966, the line was considered established and characterization of the cells commenced.

The cells presented a variegated morphology of large, spindle-shaped to polygonal and often multinucleated giant cells (Fig. 4). Special stains demonstrated absence of glycogen, mucopolysaccharides, reticulin, and neutral fat. However, the cells were faintly positive for sphingolipids as determined with Landing's stain.
Ultrastructural studies demonstrated the following characteristics, similar to those encountered in the cells composing the original tumor: abundant microfibrils and microtubules, dendritic projections of the cytoplasm, and occasional desmone-like bodies (Figs. 5 and 6). These findings were repeated over the course of the past 5 years. In no instance was ultrastructural evidence of viral particles noted.

The chromosome number always ranged from 77 to 137, with a modal number of 100 to 103. Each of the cells analyzed presented at least 2 and sometimes 3 long marker chromosomes (Fig. 7). Chromosome damage was evidenced by the presence of dicentric chromosomes in some of the cells and acentric fragments in all cells. These findings were noted every time chromosomal analysis was carried out.

The doubling time analyzed from growth curves was 63 ± 3 hr (Chart 1), and the mitotic index was 1.8.

The results of pulse-chase experiments with tritiated thymidine are depicted in Chart 2. Calculations of the periods of the cell cycle performed at the level of 50%-labeled metaphases revealed the following values: generation time, 58 hr; G1, 27.5 hr; S, 22 hr; and G2, 8.5 hr. The calculated mitotic time is 1 hour.

The percentage of labeled nuclei in continuous labeling experiments increased at a slow rate, reaching a plateau of 90% at about 40 hr (Chart 3).

Pulse labeling of exponentially growing cells demonstrated that 30 to 35% are in S phase.

DISCUSSION

As far as we are aware, this is the first established human cell line of a sarcoma of neurogenic origin. The only other mammalian neurosarcoma is the feline line reported by Noronha and Lee in 1969 (9). The line reported in the present paper is undoubtedly a neurofibrosarcoma. The ultrastructural features of Schwann-like envelopes, microfibrils, and desmoseme-like bodies are characteristic for neurosarcomas (6). Some evidence of specific lipid synthesis was demonstrated with Landing's stain, but definitive demonstration of sphingolipids awaits more sophisticated tests. The cells have maintained these morphological characteristics throughout their entire in vitro life.

Although the broad range in chromosomal number indicates that the line is polyclonal, the presence of the same marker chromosomes in all of the cells strongly suggests a common clonal origin. The high ploidy (near-triploid to near-tetraploid) may represent in vitro adaptation, since high aneuploidy is characteristic of many cell lines maintained in culture, whether of malignant or benign origin (5, 12).

The long doubling time of the present line is not unusual for mammalian cells since other cell lines present kinetic parameters of the same order (3).

The generation time is equally prolonged. By mathematical analysis, interrelating the doubling time and the cell generation time (3), it can be shown that the proportion of cells in the culture that can divide is 0.91. It is also apparent from the continuously labeling experiments that about 90% of the cells are in an actively proliferating pool. The remainder may represent cells with a longer G1 phase than the average population or cells pertaining to a G0 pool. The techniques utilized do not allow further discrimination.

Sufficient evidence has accumulated to establish this cell line, hereafter designated T2, as an in vitro model for sarcomas of neurogenic origin. We are presently utilizing T2 cells to search for the presence of a tumor-specific antigen common to sarcomatous neoplasms. Preliminary results with human sera and peripheral lymphocytes from patients with a variety of sarcomas indicate that this cell line may, in fact, possess a common sarcoma tumor antigen (13).

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REFERENCES


Fig. 1. The histological pattern of the original tumor is depicted in this photomicrograph. H & E, × 400.

Fig. 2. Electron photomicrograph demonstrating the characteristic infoldings of the membrane (original tumor). × 30,000.

Fig. 3. Phase microscopy photograph displaying the morphological aspects of the original explants. × 480.

Fig. 4. Morphological characteristics of cultured neurosarcoma cells. Wright's stain, × 480.
Fig. 5. Electron photomicrograph revealing abundant fibrils in the cytoplasm and membrane infoldings (tissue culture). X 18,200.
Fig. 6. Enlargement depicting details of the mass of fibrils. X 31,200.
Fig. 7. Karyotype of T2 cells. M, marker chromosomes; arrows, extra chromosomes.
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