A New in Vitro Cell Line Established from Human Large Cell Variant of Oat Cell Lung Cancer

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ABSTRACT

A new tissue culture cell line, SHP-77, has been established from explant cultures of a primary human lung cancer. Although the latter exhibited histological features of the so-called large or polygonal cell undifferentiated variant, neurosecretory granules were detected ultrastructurally in cells of the original tumor, culture line, and neoplasms developing after transplantation of the latter in nude mice. These features attest to the identity of the original tumor as a large-cell variant of oat cell cancer. In addition, electron microscopy revealed the presence of gland formation and intracytoplasmic lamellar bodies in the cells of SHP-77. This information indicates the potential for varied differentiation and morphological expression of so-called undifferentiated lung cancer cells and is pertinent to nosological considerations concerning human lung cancer. The cell line has maintained its morphological, karyotypic, chromosomal, and growth characteristics after transplantation to nude mice. Because of this stability, SHP-77 appears to represent a propitious cell line for in vitro and in vivo biological and therapeutic studies of this type of lung cancer.

INTRODUCTION

A number of tissue culture cell lines of human lung cancer have been established. Reports of these have been concerned principally with methodology and descriptive features of the cultured cells. Many of the primary cultures were obtained from metastatic sites rather than the primary tumor. It is of interest that only two successfully cultured cell lines from a primary oat cell variant of human lung cancer have been described, both by Japanese investigators (6, 11). The most completely described line (OAT-1975) is that recently furnished by Ohara and Okamoto (11). Pertinent chromosomal, ultrastructural, and cell inactivation analyses were performed. Suspensions of cultured cells were also successfully grown when transferred to nude mice and cheek pouches of conditioned Syrian hamsters. However, no comments concerning the ultrastructural, chromosomal, or growth characteristics of the transferred cells growing in vivo were made.

We have recently established a tissue culture cell line, designated SHP-77 (Shadyside Hospital, Pittsburgh, Pa.) which was derived from an undifferentiated, large-cell variant of lung cancer that was removed from a 54-year-old Caucasian male patient with O+ blood type. No clinical manifestations or laboratory evidence of hormone production were present. He succumbed 2 months after pulmonary resection. Suspensions of the cultured cells exhibited growth in nude mice. Tumors from the latter were successfully grown in tissue culture. The commonality of chromosome constitutions and light and electron microscopic features, as well as kinetics of growth in the cultures obtained before and after animal passage, indicates the suitability of this cell line for contemplated therapeutic studies in vitro as well as in vivo.

It was also observed that, despite the undifferentiated histological appearance of the primary tumor, neurosecretory granules could be recognized in cell cytoplasms. These were more apparent in the cultured cells and those obtained from the nude mice than in the original tumor. This, as well as other information, prompts comments relevant to the nosological identity of this histological type of human lung cancer.

MATERIALS AND METHODS

Original Tumor. A firm, tan, circumscribed but not encapsulated neoplasm measuring $4 \times 3 \times 2.5$ cm was encountered in the apical portion of the upper lobe of the resected left lung of a 54-year-old man. Its cut surface revealed foci of hemorrhage and necrosis.

Portions of the neoplasm and 7 hilar lymph nodes were fixed in Zenker's acetic fluid, dehydrated, and imbedded in paraffin. Sections of these were stained with hematoxylin and eosin, as well as the periodic acid-Schiff technique, with and without antecedent diastase digestion for examination by light microscopy.

Aliquots of the tumor were also minced into 1-mm cubes and fixed in 3% glutaraldehyde in phosphate buffer for 3 hr, rinsed in buffer, and postfixed in 1% osmium tetroxide. After dehydration, blocks were imbedded in Maraglas. Ultrathin sections were stained with lead citrate and uranyl acetate and examined by electron microscopy.

Cells from solid portions of the tumor were also immediately teased into cold Hanks' balanced saline solution containing antibiotic and anti-pleuropneumonia-like organism solution [Grand Island Biological Co. (Gibco) Grand Island, N. Y.)] according to the method of Lasfargues and Ozzello (10). After centrifugation, the cells were resuspended in Roswell Park Memorial Institute Medium 1640 that contained 10% virus-screened fetal calf serum and antibiotic and anti-pleuropneumonia-like organism solutions (Gibco) and seeded into Falcon tissue culture flasks. After incubation at 37° in a 5% CO₂ humidified atmosphere for 3 hr, the epithelial cells were then decanted with the media and reseeded in new flasks at a concentration of $2 \times 10^5$ cells/flask. The medium was changed 3 times weekly. Subculture (1:4) was accomplished at 2-week intervals by trypsinization and agitation of the flasks. Phase microscopy was continuously performed on all cultures, and aliquots from the third, sixth, and 10th subcultures were prepared.
for ultrastructural examination according to the method of Brinkley et al. (2) as outlined above.

**Estimation of In Vitro Growth.** Cells obtained from the 20th subculture (40 weeks after explantation) were counted in a hemocytometer, and dilutions were prepared so that each 2 ml of medium contained $2 \times 10^8$ viable cells as determined by trypan blue exclusion. After seeding, samples were obtained at 48-hr intervals and the number of cells in each of 2 flasks was estimated by the average of 10 counts with a hemocytometer. A mitotic index was estimated by counting the number of mitoses per 500 cells from cultures prepared on glass-slide flasks (Flaskettes, Lab-Tek) and stained by the Papanicolaou method after fixation with ethanol.

**Chromosome Analyses.** Proliferating cells in the log phase of growth obtained from the 18th subculture were arrested in metaphase by adding Colcemid, 0.1 $\mu$g/ml, to the medium. Cells were detached by trypsinization and agitation and swollen by exposure to 0.075 M KCl for 10 min. Cells were then fixed in methanol:glacial acetic acid (3:1) and spread on slides. Chromosome banding was performed according to the G-banding method of Klinger (8) and interpreted according to guidelines established by the Paris Conference (12).

**Assessment of Culture Contamination.** Periodic monitoring of cultures in mycoplasma agar medium (Gibco) was performed for the detection of possible mycoplasma contamination.

The possibility of HeLa cell contamination was determined by G6PD* isoenzyme analysis according to the method of Kirkman (7).

**Transfer of Cultured Cells to Nude Mice.** Six male and 6 female congenitally athymic BALB/c mice (A. R. S. Sprague-Dawley, Madison, Wis.) that were maintained in a pathogen-free environment with sterile food and water ad libitum received a s.c. inoculation of $1 \times 10^6$ viable cells obtained from the 18th subculture of SHP-77.

Portions of the tumors obtained after growth were prepared for light and electron microscopy, tissue culture, in vitro growth, and karyotypic analysis as described above for the original tumor.

**RESULTS**

**Original Tumor.** Sections of the lung tumor revealed a malignant epithelial neoplasm comprised of polygonal, columnar, and round cells with modest amounts of acidophilic cytoplasm. Nuclei were large and for the most part ovoid or round with focally dense chromatin and occasional prominent nucleoli. Mitoses, including atypical forms, were frequent. The tumor cells were arranged in clusters, large groups, and islands, and in some foci ribbons or festoons (Fig. 1). Evidence of gland formation, tubules, or keratinization was lacking. Perineural space invasion was present. The epithelial aggregates were separated by varying amounts of relatively acellular fibrous stroma containing thin-walled blood vessels. Large areas of tumor necrosis were evident. The neoplasm appeared to arise within the bronchial mucosa, and focal dysplasia of the epithelial layer of this latter was present. No periodic acid-Schiff-positive material was identified within the neoplastic cells. Four of 7 hilar lymph nodes exhibited foci of metastases.

Electron microscopy revealed tumor cell cytoplasms generally to contain abundant swollen mitochondria with few internal cristae. Rough endoplasmic reticulum was frequently dilated with invaginations of cell cytoplasm (Fig. 2). In some cells the rough endoplasmic reticulum was linear and in rarer instances disclosed an annulate configuration. Ribosomes were frequently present in cell cytoplasms. Delicate cytofilaments and microtubules were rarely encountered. However, no tonofilaments or mucin droplets were noted. Some but not all cells contained membrane-bound, homogeneous, dense-core cytoplasmic bodies with an average diameter of 0.16 $\mu$m. Some of these bodies contained a lucid zone between their membrane and core. Other cytoplasmic bodies exhibited the characteristic appearance of lipofuscin and other lysosomal elements. Golgi structures were sparse. Nuclei often exhibited an irregular border and contained clumps of chromatin as well as commonplace nucleoli. Cell membranes of adjacent cells were occasionally punctuated by junctional complexes of the macula adherens and occludens types.

**Cultured Tumor Cells.** SHP-77 has been maintained continuously since July 1977. The culture first grew on plastic flasks as suspended or attached grape-like clusters of round and refractile cells. Transition to a monolayer form of growth was gradual, becoming dominant at about 5 months. Cultures are now characteristically comprised of islands of adherent, finely granular, round and polygonal cells (Fig. 3).

Ultrastructurally, the cultured tumor cells contained only a modest number of pleomorphic mitochondria. The rough endoplasmic reticulum was for the most part narrow and linear. Ribosomes as well as polyribosomes were frequent. Golgi complexes were also distinct in many cells. Occasional myelin figures were noted and some cells contained congeries of dense bodies. Most of the latter exhibited a homogeneous dense core surrounded in turn by a lucid halo and membrane (Fig. 4). The average diameter of these bodies was 0.15 $\mu$m. Other cytoplasmic inclusions exhibited features of lipofuscin and other forms of lysosomes. Cell nuclei were round with frequent forms exhibiting an irregular border. Chromatin appeared for the most part uniformly dispersed, and nucleoli when present were not unusual. Opposing cell membranes occasionally contained junctional complexes of macula adherens and occludens types. Clusters of tumor cells often exhibited a central lumen, and microvillus transformation of the cell membranes abutting such spaces was apparent (Fig. 5). Cannibalization of tumor cells was occasionally encountered.

The characteristic growth rate of SHP-77 is shown in Chart 1. In the log phase of growth, the doubling time was found to be 85 hr. The mitotic index averaged 1.8%.

A histogram of chromosome counts of 100 SHP-77 cells disclosed a modal chromosome number of 54 (Chart 2). Karyotypic analysis of 50 banded metaphases disclosed variations. However, all cells examined revealed a large submetacentric chromosome and lacked an intact chromosome no. 1. Some less obvious markers were also uniformly present, whereas others were inconsistent. A Y chromosome was noted in most cells (Fig. 6).

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* The abbreviation used is: G6PD, glucose 6-phosphate dehydrogenase.
No evidence of mycoplasma contamination was encountered as revealed by negative cultures and the failure to detect these organisms in the many electron micrographs studied.

Isoenzyme analysis revealed type B (slow-moving) G6PD.

Transplanted Tumors in Nude Mice. One male and 2 female nude mice developed tumor nodules at the site of inoculation. Two appeared first at 28 days as 2-mm nodules. They measured 8 mm at Day 50. They were removed for pathological, karyotypic, and kinetic studies. The other was observed first at 56 days and exhibited a growth pattern similar to that of those occurring earlier. This tumor is being utilized for transplantation into other nude mice.

Light microscopy revealed that s.c. transplants exhibited histological features that were, for the most part, indistinguishable from those of the primary tumor (Fig. 7). The tumor appeared circumscribed but not encapsulated, with only a slight degree of infiltration of the surrounding adipose tissue. Tumor necrosis, unlike that in the primary tumor, was not observed. A lymph node adjacent to one of the tumors disclosed marked sinusoidal dilation and focal cortical fibrosis and histiocyte infiltration but no evidence of metastasis. No visceral metastases were observed in the nude mice at the time they were killed.

Ultrastructurally, the tumor was comprised of light and lesser numbers of dark cells, the latter representing degenerative forms. Cell cytoplasmas contained a modest number of round swollen mitochondria with relatively few delicate cristae. Rough endoplasmic reticulum was linear as well as dilated. Papillary cytoplasmic invaginations were noted in the latter (Fig. 8). Free ribosomal particles and polyribosomal aggregates and an occasional Golgi apparatus were present. Dense core secretory granules similar to those noted in the primary tumor and rare lysosomes and lipofuscin bodies were also present. Cell attachment plates of macula adherens and occludens types were evident, and in some instances adjacent cells exhibited a somewhat scalloped border due to pseudopodal extensions of one cell into the adjacent member. Nuclei exhibited an irregular or round border with small clumps of chromatin. Nucleoli were of the usual type.

Tissue culture characteristics of the transplanted tumor were similar to those of the primary tumor. Comparison of distribution of chromosome counts in 100 cells revealed that the modal chromosome number of 54 was maintained. Marker forms and Y chromosomes were similarly distributed.

DISCUSSION

Examination of the cell line established as SHP-77 has provided pertinent information relative to the cytogenesis of lung cancer. Histopathological classification of the original tumor as well as those transplanted from SHP-77 into nude mice, from which it was found to be morphologically indistinguishable, might be regarded as debatable if not difficult. These neoplasms exhibited such light microscopic features of oat cell carcinoma as marked anaplasia, frequent mitoses, and a tendency for the formation of ribbon-like aggregates. Yet, their relatively large size, frequent polygonal shape, and modest amount of acidophilic cytoplasm might also, according to prevailing criteria, warrant a diagnosis of undifferentiated, large, or polygonal cell carcinoma. It is this type of lung cancer that is frequently the source of interobserver disagreement. There is accumulating evidence, recently substantiated by the electron microscopic studies of Churg (3), which strongly implies that such undifferentiated carcinomas represent either poorly differentiated squamous cell or adenocarcinoma types. However, none of the cells from the original or transplanted tumors or those of the cell line contained tonofilaments and other characteristics indicative of squamous cell carcinoma. On the other hand, all material examined by electron microscopy revealed that varying numbers...
of cells possessed dense core granules of the neurosecretory type, being in this regard consistent with their oat cell identification (1, 5). In addition, lumen formation was not uncommon in electron microscopic preparations of the cell line. This latter criterion, despite the failure to demonstrate mucin granules in electron microscopic preparations or tinctorially, is generally held as sufficient for the diagnosis of adenocarcinoma (3). Furthermore, the cultured cells also occasionally contained concentric whirls of osmophilic membranes reminiscent of the lamellar bodies of type II pneumocytes demonstrated by Coalson et al. (4) in those pulmonary cancers considered to be of the bronchoalveolar type. Yet, the true identity and significance of these intracytoplasmic inclusions are tempered with the realization that comparable-appearing structures represent a relatively common "artifact" of cultured cells (13). Nevertheless, it is apparent that "undifferentiated" cells comprising pulmonary cancers may possess and indeed subtly exhibit the capability of varied differentiation and morphological expression. In this light one might justifiably conclude that the extent classification (9), as well as recent studies concerning the nosological position of so-called "undifferentiated" carcinoma of the lung, may be too intransient. The above considerations also prompt us to regard this lung cancer as a variant of the oat cell type. It is of interest in this regard to note that Spencer (14) briefly refers to such a large-cell histological variant of oat cell cancer.

SHP-77 differs from the established cell line of oat cell carcinoma (OAT-1975) recently described by Ohara and Okamoto. The original tumor, which is utilized as well as transplants to nude mice, appears at least from the pertinent photographs, to represent the small-cell or more classical form of oat cell cancer of the lung. Interestingly, these investigators failed to find neurosecretory granules in cultured cells, although such inclusions were noted in electron microscopic preparations of the original tumor. This dichotomy was attributed to the vigorous growth of the cells in vitro with a resultant loss of functional stability. Furthermore, they provide no information concerning the chromosomal characteristics of transplanted tumors growing in nude mice. In regard to the latter, it is of interest that we obtained only 3 of 12 successful transplant takes, whereas Ohara and Okamoto (11) observed all cell transplants into nude mice to be successful and to exhibit earlier growth, although similar numbers of cells were inoculated. Identical karyotypic features of the neoplastic cells in the established cell line and cells cultured from the transplant were encountered in our experiments. The chromosomal characteristics of OAT-1975 and SHP-77 were also divergent. OAT-1975 contained cells with a hypertriploid modal number, whereas that of cells in SPH-77 was 54.

Data obtained in this study clearly eliminate the possibility of HeLa contamination of SHP-77. HeLa cells have never been brought into or propagated in our laboratory. The presence of Y chromosomes, type B G6PD isoenzyme, and divergent markers from those noted in HeLa substantiate our conclusion in this regard.

The persistence of the integrity of the structural, karyotypic, and growth characteristics of SPH-77, not only in culture but after in vivo transplantation into nude mice, indicates that it represents a stable and propitious cell line for biological, immunological, and therapeutic studies of this type of tumor.

Although oat cell cancer of the human lung has a marked propensity for metastases, such an event was not observed in the nude mice with transplanted tumors. Whether this divergence in behavior is related to the site of transplantation or duration of tumor growth is now being investigated.

REFERENCES

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