The Establishment of a Cell Line of Human Hormone-synthesizing Trophoblastic Cells in Vitro

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SUMMARY

A human hormone-synthesizing trophoblastic cell system has been established in vitro and may prove to be the first functional human embryonic cell line in continuous culture. Chorionic gonadotropin hormone produced by these cultures serves as a marker for identification of the trophoblastic cell. No interruption in this property nor change in cytologic display has occurred during 1.5 years in continuous culture. The continued proliferation of the undifferentiated cytotrophoblast is being stabilized in serial cultivation.

INTRODUCTION

The establishment of functional human endocrine tumors in continuous tissue culture has met with no previously reported successes. Because of the lack of means to identify and select functional cell types for culture, it has been generally true that organ-specific function becomes rapidly deleted in vitro. Functioning tumorous rat and mouse cultures of endocrine origin have been recently reported by Yasumura et al. (13) with the use of an alternate animal-to-culture-to-animal-to-culture serial passage technic.

A human system suitable in many respects for in vitro propagation is present in the complex multifunctional endocrine organ, the placenta. Many of the properties of the parenchymal trophoblast cells of the placenta have been compared with those of cancer cells (12). With regard to differentiation, a key consideration in neoplasia, the biologic history of the trophoblast can be traced to the first order of differentiation of the human ovum occurring sometime before the third or fourth day after fertilization. At this time, the first order of specialization of cells of the cleaving morula was observed by Hertig and Rock (7) in the 58 cell human ovum when two cell types first appeared, embryonic and trophoblastic. By present assay methods, the pregnancy gonadotropin (human chorionic gonadotropin) is detectable at 7 days (6), though direct sampling of ovum fluid would probably make this possible at the time of this first order differentiation. Thus, glycoprotein hormone production by trophoblastic cells is the first detectable hormonal specialization to occur in a human cell. No other cell performs this function normally and, therefore, chorionic gonadotropin-producing tumors are expressions of trophoblastic cell function.

Functionally, this cell produces an easily detected hormonal marker, and it represents the first step of the ladder of differentiation. Furthermore, in its malignant form it comprises the first malignancy to be successfully treated by chemotherapy (9). These factors make it an ideal model for attempts at isolation and propagation in vitro.

MATERIALS AND METHODS

Human choriocarcinoma, taken at autopsy from a cerebral metastasis, was previously transplanted to the cheek pouch of the hamster by Dr. Roy Hertz (8) and maintained through 304 serial transfers over a period of 8 years. Such a tumor was removed from the cheek pouch for attempts at establishment in vitro. Fresh specimens of choriocarcinoma from surgical or autopsy cases were obtained when available.

Nutritional Factors

An initial consideration of nutritional requirements of the malignant trophoblast was based upon growth response observed with the normal placenta in vitro (10). Periodic acid-Schiff stains on the advancing columns of cytotrophoblast of the normal ovum occurring sometime before the third or fourth day after fertilization. At this time, the first order of specialization of cells of the cleaving morula was observed by Hertig and Rock (7) in the 58 cell human ovum when two cell types first appeared, embryonic and trophoblastic. By present assay methods, the pregnancy gonadotropin...
Matrix

Normal and malignant cells have been shown to have increased growth potential when explanted on a collagen matrix (2). Since the original in vivo cellular framework consists of varying proportions of collagen, reticulum, fibrin, and other acid mucopolysaccharides, the use of reconstituted tropocollagen prepared by a modification of the method of Ehrmann and Gey (2) was undertaken. Also, human fibrinogen clotted with thrombin was employed in some cases.

Conditioning and Parabiosis

Interactions of cells of different types to provide necessary growth factors has been well established (5). The use of conditioned medium or the establishment of parabiotic cultures to serve as "feeder layers" for the stimulation of new growth has also proved beneficial. The utilization by one cell of the product of another serves important functions in creating appropriate mesenchymal-epithelial interactions in vivo and in vitro.

Because the early history of the normal trophoblast is intimately associated with a decidual bed rich in glycogen, for the probable purpose of nourishment of the implanting blastocyst in the process of placentation, it was deemed advisable to attempt to enhance growth of choriocarcinoma by primary explantation of the tumor in a parabiotic culture with decidua explanted from a normal pregnancy several days prior.

Observations of Cultures

The roller tube method of culture (3) was used with constant temperature microscope chambers to facilitate daily and extensive study of the cultures. Direct microscopic observation of the living cells through the roller tube using an upright microscope enclosed in the incubator chamber made it possible to study the cultures extensively and perform the indicated operations. These operations included the appropriate microsurgical operations to select and to isolate the desired cells for further cultivation and the elimination of fibroblasts. Other operations required segregation of the cocultures and modification of components of the medium as indicated by morphologic manifestations of growth observed through daily examinations.

Tissue Fluid Administration

Because of the elevated metabolism possessed by these cells and a high level of CO₂ production, a rapid drop in pH was noted. It was, therefore, generally necessary to exchange the tissue culture fluid every day.

Preparation of Collagen Tubes

Collagen tubes were prepared originally as slants of 1.0 ml of reconstituted tropocollagen. The collagen gel was always equilibrated in the final step with the culture medium. It was soon noted that collagen lysis was produced by these cell cultures, causing defects in the gel with subsequent contractions and retractions of the tissue culture cell mass along peripheral lines of force. Because of this defect, the collagen preparation was modified by spreading only a thin film of collagen on the surface of the roller tube sufficient for cellular attachment but apparently insufficient to result in an open gap because of bulk collagen extraction when lysis occurred.

Selection of Tissue for Culture

Because of the nature of growth of choriocarcinoma, a difficult problem was encountered in selecting viable tumor cells from the mass of blood and necrotic areas characteristic of this tumor. Previous histologic experience has shown that only those cells on the peripheral advancing margins of a tumor nodule are viable since no stroma with its accompanying vascular supply is found in this tumor. Hematoxylin and eosin sections of the tumor revealed vascular lacunae similar to those found in the early blastocyst; however, no apparent organized pattern exists and early necrosis ensues. To overcome this problem, fragments of tumor 3-4 mm in size were divided in half; one-half were kept sterile in the Petri dish and the other half were quick frozen in the cryostat, sectioned at 6 microns, stained, and examined for viable tumor cells. Some improvement in selection could be achieved in this way; however, the available viable tumor cell mass remained undesirably minimal in quantity—of the order of only a few percent of a given tumor nodule.

Explantation of Primary Cultures

Surgical and autopsy specimens of choriocarcinoma were obtained through the cooperation of a number of physicians and treatment centers for patients with this tumor. All patients had had recent courses of Methotrexate, some also with actinomycin D. The tumor specimens were usually transported to the laboratory in the nutrient medium at 4°C. A time interval of 8 to 48 hours elapsed between the time of excision of the tumor and the time of explantation in tissue culture, except in the case of the specimen from which the BeWo cell line was established. This was taken directly from the hamster cheek pouch and explanted immediately in culture at 37°C. Fresh tumor specimens were sent from Tokyo, Milwaukee, Boston, New Jersey, New Orleans, and Texas.

The tumor specimens were dissected in sterile glass Petri dishes containing small pools of growth media. After appropriate selection, the tissue was cut into approximately 1 sq mm fragments using crossed scalpels with #11 Bard-Parker blades. Four to six of these fragments were transferred to 16 x 150 mm collagen roller tubes containing 1 ml of growth medium. After standing in the incubator for 15 minutes to allow for adhesion to the collagen, the culture tube was then rotated in the rollerdrum.

RESULTS

A total of 7 choriocarcinoma specimens were explanted in culture. Microscopically identifiable tumor cell growth occurred in the primary explants of 3 specimens. Small numbers of apparent tumor cells were maintained from two of these specimens for 4 months in tissue culture; however, collagen lysis and retraction ultimately disrupted and dislodged the very small tumor colonies with ultimate loss of these cells. The others showed few indications of growth potential when received.
The tissue from which the cell line was established was the specimen which had previously been serially transplanted in the hamster cheek pouch in Doctor Hertz's laboratory. From this specimen several experimental designs were used including cultures explanted as cocultures with human decidua, cultures explanted on collagen matrix alone, on human fibrin matrix, and on collagen plus fibrin. Cultures were also explanted with placental cord serum, adult maternal serum, and calf serum.

From twenty-six roller tube cultures explanted in three separate experiments using combinations of the above design, one tumor colony from a single culture has given rise to the now established cell line BeWo. It has been in continuous cultivation for 1.5 years. This colony was derived from the parabiotic combination of choriocarcinoma and human decidual tissue explanted in culture from the endometrium of a normal pregnancy explanted 48 hours previously. The choriocarcinoma was explanted in the same culture tube with the already proliferating decidua imbedded on a collagen matrix with medium consisting of 70% Waymouth's MB 752/1 and 30% placental cord serum.

Within 24 hours migration of a heterogeneous population of many cell types was observed. Among these, which included decidual cells, broad irregular spindle cells, macrophages, and fibroblasts, was a thick aggregate of grayish-white cystic cells which had previously been noted to be characteristic of cytotrophoblast cell aggregates (cell column cytotrophoblast) of the normal placenta in vitro (10). When these cells migrated from the colony, they displayed multiple extended processes giving an asteroid or burr-like appearance described by Gey (4) in early trophoblast tissue cultures. The medium from this culture was extracted and assayed for chorionic gonadotropin by the immature rat uterine weight method of Delfs (1). The assay proved strongly positive.

Attempts were then made to segregate and isolate from the multiple cellular array the portions of the colonies containing masses of tumor cells (Fig. 1). This was accomplished with microsurgical dissection by repeatedly excising and discarding fibroblastic and other nontumorous areas. This led to homogenous populations of trophoblast after a period of several months (Fig. 2). The bioassays on fluid from these subcultures were likewise positive.

An initial rapid growth during the first 2 weeks was followed by several months of less active growth, during which time an adjustment of the medium from 70% Waymouth's MB 752/1 and 30% cord serum was changed to 50% Waymouth's MB 752/1, 40% Gey's balanced salt solution, and 10% cord serum. This change resulted in the elimination of diffuse granules from the cytoplasm believed to reflect the excessively high glucose levels resulting in excess glycogen storage as seen by periodic acid-Schiff staining.

Adaptation to growth on the more abundantly available newborn calf serum has now been accomplished. The necessity for a collagen matrix was eliminated by gradual adaptation to growth on glass and to Falcon plastic flasks accomplished over a 5-month period of time. Attempts at more rapid adaptation failed.

Subcultures are performed on a weekly basis by mechanically disaggregating the colonies and subculturing on a 1 to 4 basis. The rapid outgrowth still necessitates media renewals each day.

Chromosome analysis of squash preparations reveal a highly aneuploid human cell line. A modal number of 86 chromosomes was obtained in 100 cells counted (Fig. 3).

**Hormone Assays**

The synthesis of the glycoprotein hormone of pregnancy, human chorionic gonadotropin, represents a fundamental property of the trophoblast. The level of hormone production in duplicate 30-ml Falcon flasks was determined by quantitative bioassay. The colonies in the flasks were treated with 0.25% trypsin in balanced salt solution free of Ca++ and shaken for 10 minutes at 37°C. After filtering through gauze to remove mucus debris, triplicate counts of the cells were made on the Coulter counter. Average counts yield about 1.3 million cells, producing approximately $1 \times 10^{-5}$ international units of gonadotropin per cell in a 24-hour period. Quantitative bioassays were employed after the cell line had been in continuous propagation for approximately 16 months. Qualitative bioassays (Delfs' method) and immunoassays (Wampole hemagglutination inhibition) done at approximately monthly intervals throughout the culture history were consistently positive.

In addition to preservation of functional hormone synthesis in the BeWo line, there has also been a preservation of cell morphology of the cytotrophoblast of the original tumor (Figs. 4, 5).

**DISCUSSION**

The most characteristic function of the undifferentiated trophoblast cell is the production of the chorionic gonadotropin hormone. This hormone is produced by the trophoblast of the normal placenta and by its malignant derivative, choriocarcinoma. This biologic marker provides a convenient means by which hormone synthesis, differentiation, response to chemotherapy, nutritional requirements, and metabolic pathways are being evaluated in vitro.

**REFERENCES**

Roland A. Pattillo and George O. Gey

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Fig. 1. Zone of tumor growth on the left with sharp boundary between tumor, an acellular intermediate zone, and fibroblast on the right superior. × 150.

Fig. 2. Segregated cultures of cytotrophoblast. Extracts of the medium in which these cells are grown give positive assays for the gonadotropin hormone. × 150.

Fig. 3. Aneuploid karyotype of line BeWo performed after 8 months in tissue culture.

Fig. 4. Hematoxylin and eosin section of original tumor from which strain was derived. Predominant cell is cytotrophoblast with large vesicular nuclei. Occasional syncytial formation seen in darker, tapering, ellipsoid configurations. × 500.

Fig. 5. Hematoxylin and eosin section of a colony from tissue culture tube. The morphology of the cytotrophoblast is maintained in continuous culture. × 500.
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