The Tissue Culture and Morphology of Human Breast Tumor Cell Line BOT-2

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SUMMARY

A continuous human breast tumor cell line (BOT-2) was derived from an infiltrating duct carcinoma. The tumor cell line was grown as a monolayer in flasks, but the cells could be readily adapted to growth in roller cultures. These studies indicate that BOT-2 cells have a 16- to 18-hr doubling time and a modal chromosomal number of 63. The original BOT-2 cell culture has been in continuous cultivation for almost 2 years and has been passed 137 times. The BOT-2 cell line has been differentiated from HeLa cells by isoenzyme studies and chromosomal analysis.

INTRODUCTION

Human breast tumors have been notoriously difficult to establish in vitro as continuous cell lines. Most of those breast tumor cell lines that have been maintained for long periods are either from metastatic sites or pleural effusions. Although many modifications of in vitro techniques, such as serum replacement and additions of estrogen, insulin, glucose, etc., have been tried, most tumor cell cultures derived from the original tumor sites have been maintained for only 3 to 6 months; to our knowledge there are only a few reports of explant-derived human breast tumor cell lines that proliferate after extended periods in culture (1, 4, 9, 11). However, due to the current trends of investigation in the field of tumor immunology, it is necessary to have continuing in vitro representations of malignant cells from primary tumors for the assay of patient tumor immunity.

This paper describes a human breast tumor cell line, BOT-2 (Breast Original Tumor), that was derived from an infiltrating duct carcinoma surgically removed from a 31-year-old Caucasian female who had no previous clinical treatment. The BOT-2 cell line was shown to differ from HeLa cells by the presence of the “B” type G6PD isoenzyme pattern and by dissimilarity of the alkaline phosphatase isoenzyme pattern. A 2nd difference between BOT-2 and HeLa was the modal chromosomal number and karyotype pattern.

MATERIALS AND METHODS

Tissue culture. Tumor tissue obtained at surgery was immediately immersed in Eagle’s minimum essential medium with Earle’s salts. Additives in this medium (hereafter called complete medium) were gentamycin (100 µg/ml), 20% heat-inactivated fetal calf serum, and 1 mM glutamine (Grand Island Biological Co., Grand Island, N. Y.). The tissue was then cut into small pieces with surgical scissors and minced with sterile razor blades until the explant size was less than 1 cm. The explants were transferred to flasks that had been preconditioned with a thin film of heat-denatured fetal calf serum. These flasks were prepared by coating the surface with a thin film of serum and allowing them to sit in the hood for 30 min. The explants were then covered with complete medium and incubated in an atmosphere of 5% CO₂ and 95% air at 37°C. In our experience, cultures of breast tumors were more likely to be successful if they were set up as described and remained undisturbed for periods up to 3 weeks with a minimum of handling and no gassing or medium change.

During these studies we have observed that primary explants from breast tissue achieve better growth if they are allowed to float freely about the flask. After attachment occurred, the explants were broken loose and allowed to float again. When sufficient small colonies had been established, the explants were moved to a 2nd flask, and the attached cells trypsinized with 0.025% trypsin in Eagle’s minimum essential medium to obtain a dispersed cell layer. When enough monolayers were established, cell samples were suspended in a complete medium containing 10% glycerine or 10% dimethyl sulfoxide and frozen in liquid nitrogen for storage.

Cell-doubling times in the 50th and 100th passages were measured by trypsinizing the cells and seeding 5000 cells/flask in 10 replicate Falcon T-25 flasks. Daily total cell counts were made on 2 randomly selected replicate flasks at exact 24-hr intervals for 5 days.

Tumorigenicity. Athymic nude mice were used to estimate malignancy of the BOT-2 cell lines. The animals received s.c. injections of 5 x 10⁶ cells in 0.15 M NaCl:0.1 M phosphate, pH 7.2.

Light Microscopy. Frozen sections of the original tumor tissue immediately adjacent to the cultured tumor were cut at 10 µm, fixed in 10% neutral buffered formalin, and stained with hemotoxylin and eosin. Living cells were

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2 The abbreviation used is: G6PD, glucose-6-phosphate dehydrogenase.

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Electron Microscopy. Cultured breast tumor cells were trypsinized from the flasks and fixed with 6.25% glutaraldehyde in 0.1 M phosphate buffer plus 5% sucrose (pH 7.2). The cells were then postfixed in 1% OsO4 in phosphate buffer (pH 7.2), dehydrated in graded ethanol, and embedded in Spurr epoxy resin (Polysciences Inc., Rydal, Pa.). After curing for 24 hr at 57°, thin sections were cut on an LKB-Huxley ultramicrotome and stained with uranyl acetate and lead citrate (12). The finished preparations were viewed in a Hitachi HU-11B electron microscope.

Isoenzyme Studies. As a check on possible cross-contamination of the breast tumor cell line with HeLa cell strains, the isoenzyme patterns of G6PD and alkaline phosphatase from both lines were compared in starch gel. For G6PD isoenzyme patterns, starch gels were prepared by the method of Kirkman (8), using Electro-Starch (Electro-Starch Co., Madison, Wis.). Gels were inoculated with the 37,000 x g supernatant fluid from sonically treated cells, and a current of 130 V and 30 ma was applied to the gel for 14 hr. After slicing, the gel was stained by the staining gel method described by Kirkman, with the omission of the KCN.

Isoenzyme patterns for alkaline phosphatase were determined in starch gels prepared with Electro-Starch in 0.3 M borate buffer, pH 8.3. The gels were inoculated with dialyzed 1-butanol extracts, and the electrophoresis was carried out as previously described (5). The sliced gels were stained for alkaline phosphatase isoenzymes by the histochemical staining method of Kaplow (6).

Chromosomal Studies. In view of Nelson-Rees’ paper (9) suggesting HeLa contamination of established tumor cell lines, cytogenetic analysis of BOT-2 was performed in the 116th culture passage to ensure against such contamination. Metaphase plates were obtained by the procedure of Bottomley et al. (2), with the modification that incubation in Colcemid was reduced from 4 to 1 hr in order to prevent overcontraction of the chromosomes.

RESULTS

Tissue Culture. After 2 to 3 weeks, during which the cultures were not disturbed, most of the flasks contained one or more explants that exhibited outgrowth. In some instances, the outgrowth consisted of mixed epithelial cells and fibroblasts. However, in other cases, the outgrowth appeared completely epithelial, and these cultures were selected for further work. No cells with fibroblastic characteristics have been encountered after this 1st selection. After the outgrowth was well underway, the explants that had not attached were placed in another flask, and growth was allowed to continue. When the outgrowth had become at least twice the diameter of the original explant, the explants were dislodged and allowed to attach in other areas of the flask. After several moves of the explants, the flasks contained an almost confluent cell layer. The cells could then be trypsinized (0.025% trypsin) and split 1:2, while the explants were moved to a 3rd flask.

Following trypsinization, the cells formed a smooth monolayer which eventually became thick colonies in some areas of the flask. In the 20th passage, the fetal calf serum level was lowered to 10% from the original 20% and the monolayer became much more uniform, although some clumping still occurred. Thereafter, all monolayers were grown in medium with 10% fetal calf serum. Fetal calf serum has a wide variety of hormones and contains estrogen in excess of 1000 µg/ml, so it appears that the addition of hormones is unnecessary and may be growth inhibitory.

The original monolayers of BOT-2 have been continuously cultured for almost 2 years and are now in the 137th passage.

The BOT-2 cells adapted readily to conditions in roller bottles and grew to high population densities on the standard growth medium containing 10% fetal calf serum. Doubling-time studies indicate that the BOT-2 cell line had a doubling time of 16 to 18 hr (duplicated in the laboratory of Dr. M. J. Griffin), when grown on complete medium containing 10% heat-inactivated fetal calf serum.

Light Microscopy. Frozen sections of the carcinoma immediately adjacent to the area taken for culture showed the typical picture of an infiltrating ductal carcinoma, i.e., multiple nests of epithelial cells, which were characterized by large nuclei of uneven size and morphology. (Fig. 1).

Tumorigenicity. The s.c. injection of 5 x 10⁶ BOT-2 cells into nude mice resulted in tumors. These solid tumors have now been transplanted through 4 generations of nude mice. The histological pattern of these tumors was consistent with that of the original tumor, i.e., multiple nests of cells which resemble the original human tumor (Fig. 2).

Electron Microscopy. The plasma membrane of BOT-2 cells appeared normal with a moderate amount of pinocytotic activity. Desmosomes were present in almost every cell cut. The cytoplasm contained short segments of rough endoplasmic reticulum, moderate numbers of mitochondria, and lysosomes and one or more Golgi zones. The nucleus was generally round without major lobulation and contained one or more large, well-defined nucleoli (Fig. 3).

Isoenzyme Studies. The G6PD isozyme pattern in Fig. 4 shows the breast tumor cell line to have the “B” type of isoenzyme, while the HeLa cell line has the “A” type isoenzyme.

Further differences in isoenzyme patterns were observed in gels stained for alkaline phosphatase enzyme activity (Fig. 4B). The HeLa cells had only 1 band of alkaline phosphatase activity which migrated toward the anode and was rather broad. In contrast, the breast tumor cell line had 3 bands of alkaline phosphatase activity. In addition to a band of activity that corresponded to that of the HeLa cell enzyme, the breast cell line had a band of activity that migrated faster toward the positive electrode, but remained near the inoculation site.

Chromosomal Studies. Chromosomes of 50 cells were counted, yielding a modal chromosome number of 63 (Chart 1). Thirty-two % of those cells counted demonstrated a chromosome number of 63. Karyotypes of several cells of
Fig. 1. Light micrograph of the original human breast carcinoma. H & E, × 200.
Fig. 2. Light micrograph of a s.c. cancer in a nude mouse after the injection of BOT-2 cells. H & E, × 200.

Chart 1. This graph depicts the chromosomal number distribution in a random count of 50 BOT-2 cells. The modal number was 63.

DISCUSSION

The tumor cell line described in this paper has characteristics that typify it as an epithelial cell line derived from human breast cancer and make it a worthwhile cell line to study. The BOT-2 cell line has growth and morphological characteristics that are similar to but not unique to several established breast tumor cell lines. These parameters are:

a) a 16- to 18-hr doubling time, which compares with the 18-hr interval seen in the HBT-3 cell line (1);

b) a tendency to grow in multilayered colonies, a behavior similar to that of MDA-MB-157 (13), HBT-3 (1), and HBT-39 (11); and

c) a modal chromosomal number of 63, comparable to 64 to 66 for MDA-MB-157 (13), 62 for HBT-39 (11), and 62 for CaMa (4).

In the past the lack of success with human explants may have been due to culturing techniques, for it is our experience that the more complex the medium and methodology the less likely the chance of in vitro proliferation. As was stated in “Materials and Methods,” one should not be
continually moving flasks, changing medium, and gassing to change pH. In our work, fibroblasts seem to proliferate much more rapidly in the conditions of normal culturing, i.e., frequent medium changes and trypsinization. In contrast, under the conditions described in the “Tissue Culture” methods, it is our experience that pure epithelial cultures can be obtained.

Although some authors profess the advantage of culturing breast tumor effusions (3, 13), in our studies the likelihood of establishing a tumor cell line from this material was not greater than beginning with primary tumor tissue. We have found that, while effusions often have a luxuriant growth at the outset, they are rarely characteristic of the cell type grown from primary tumor tissue, and even more rare is the effusion cell which has an infinite life-span.

Since the rationale for development of tumor cell lines is the creation of an in vitro representation of the malignant cells in the primary tumor, it seems more logical to begin with tumor cells from the original tumor site. In this way, one can at least ensure that the beginning in vitro system is as similar to the primary tumor as possible and should resemble it morphologically and immunologically. For the reasons of immunological studies, cloning of this cell line resembles it morphologically and immunologically. For the reasons of immunological studies, cloning of this cell line has not been done, because Killion et al. (7) have demonstrated in L1210 murine leukemia that antigenic subpopulations exist that have greater or lesser tumor antigenicity than the original total tumor cell population.

As a result of recent publications (10), it appears necessary to defend the source of the BOT-2 cell line from possible incrimination as a HeLa contaminant. There are several very good reasons to believe that the BOT-2 cell is a true, non-HeLa cell line: (a) HeLa cells are not grown in this laboratory; (b) the original explants of breast tumor tissue are still “living” and continue to seed new cells with identical characteristics after more than 18 months in culture; (c) the G6PD isoenzyme pattern was the “B” type; (d) the alkaline phosphatase isoenzymes have different banding patterns; and (e) the BOT-2 cells have a distinctly different karyotype and modal chromosomal number, compared with HeLa.

If the information accumulated by Nelson-Rees (10) is accurate, then the establishment of this breast tumor cell line is all the more important, for it would be 1 of 2 established cell lines that was derived from original breast tumor tissue and has not been contaminated with HeLa cells.

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Fig. 3. Electron micrograph showing the ultrastructure of the BOT-2 cell line. Arrows, desmosomes. × 15,000. Bar, 1 μm. Inset, high magnification of the plasmalemma of adjacent BOT-2 cells demonstrates the structure of the desmosomes. × 56,000. Bar equals 1 μm.
NORDQUIST: BREAST TUMOR (N=63)

GROUP A, 1-3

GROUP B, 4-5

GROUP C, 6-12, X

GROUP D, 13-15

GROUP E, 16-18

GROUP F, 19-20

GROUP G, 21-22

MICROCHROMOSOMES

Fig. 4. A. G6PD isoenzyme patterns for BOT-2 (BT) and HeLa (HL) demonstrating "B" type for BOT-2 and "A" type for HeLa. B, Alkaline phosphatase isoenzyme patterns for BOT-2 and HeLa showing 3 distinct bands in BOT-2 and only 1 in HeLa.

Fig. 5. Karyotype of a BOT-2 cell in the major mode.
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