Establishment of a Continuously Growing Cell Line from Primary Carcinoma of the Liver

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SUMMARY

A continuously growing cell line has been established from autopsy material taken from a primary liver carcinoma, a cancer which has a high incidence in Southern Africa. The cell line was initiated from multifocal areas of outgrowth in the primary culture, and adaptation to in vitro conditions was completed after 18 months. The cells resemble hepatocytes in culture, have a doubling time of 35-40 hours and a plating efficiency of 40-50%. No virus particles have been found in the cells by ultrastructural examination. The isozyme pattern and the karyology of the cells are human. The chromosome pattern is heteroploid (mean number 56) and there are marker chromosomes.


In a 13-year survey of malignant neoplasms in Mozambique, Prates reported that 43.1% of the malignancies in Black men were primary liver cancers, occurring most frequently in persons under the age of 40 years. In 1964 Oettle suggested that the presence of aflatoxins, produced by Aspergillus flavus and contaminating ground-nut harvests in Mozambique, may be of significance in the aetiology of the disease. Subsequent studies have shown a dose-response relationship between the intake of aflatoxin with foodstuffs and the incidence of primary liver cancer. However, the role of biological agents, particularly those which affect the liver, such as malaria, hepatitis viruses and arboviruses, cannot be regarded as non-contributory. The association between hepatoma and the presence of circulating hepatitis B surface antigen (HB.Ag) in the sera of patients may be significant. The fact that some hepatoma cells secrete alpha-fetoprotein (AFP) has been used for early diagnosis, and in this connection a survey of serum AFP levels in a population at risk has shown a seasonal incidence of cases. The establishment in vitro of cell lines derived from primary liver cancers was considered to be a logical approach to the laboratory investigations of the tumour. Permanent cell lines derived from these tumours would broaden the scope of investigations on primary liver cancer, since pure cell cultures could be used for a variety of studies which cannot be carried out on short-lived biopsy or postmortem tissue.

Chang established the first cell line from normal human liver but these cells cannot be distinguished from other undifferentiated tumour cell lines. Since then a number of studies on the cultivation of normal human liver cells have been published. The cultivation of human hepatoma cells has not been as extensively studied, although they have been maintained in culture, without passage, for two months. Chen established the first hepatoma cell line and Fogh and Trempe have reported an epithelial cell line derived from ascitic effusion from an adenocarcinoma of the liver. Prozesky et al. established a cell line from needle aspiration material from a patient with primary liver cancer, and the isozyme patterns and karyology have shown patterns characteristic of human liver and hepatoma tissue. Recently Doi reported the establishment of two cell lines from an undifferentiated human hepatocellular carcinoma, each cell line being morphologically distinct. The epithelial cell line was shown to produce AFP during the first 6 months of culture, after which this function ceased.

We report here the development and some characteristics of a continuously growing cell line obtained from a primary liver cancer.

MATERIALS AND METHODS

The patient was a Shangaan male, aged 24 years, from Mozambique. He was admitted to hospital in March 1973. AFP and HB.Ag were measured in his serum. At autopsy in June 1973 the following specimens were taken for explant in vitro: (i) from the primary tumour site; (ii) from a secondary distant tumour in the liver; and (iii) from a secondary tumour in the lung. Small portions from each specimen were removed for histology and electron microscopy, while the remainder was prepared for tissue culture.

Tissue Culture

Each specimen, approximately 1 cm in size, was transported to the laboratory in separate jars containing sterile growth medium consisting of minimum essential medium with non-essential amino acids (Grand Island Biological Co., NY), 10% heat-inactivated fetal bovine serum (GIBCO), 100 µg/ml streptomycin and 100 U/ml penicillin, to which an extra 100 µg/ml Mycostatin was added. Each specimen was washed twice with growth medium and minced with scissors into 0.1-1 mm³ pieces. Each specimen was divided into three samples; one was softened by trituration through a 14-gauge needle with growth medium.
and then planted into 25-cm² tissue culture flasks (Falcon Plastics) at 1,5 ml per flask. The second samples were incubated for 5 minutes with 0,25% trypsin in phosphate buffered saline (PBS), and the third samples were trypsinised for 20 minutes. All trypsinised samples were centrifuged at 1 000 g for 5 minutes, washed once with growth medium, and planted in the same manner as the non-trypsinised medium samples. All flasks were flushed with CO₂ to adjust the medium to pH 6,8 and incubated at 37°C. The flasks were examined after 2 days for cellular attachment and growth medium was replaced with 5 ml of fresh medium, which was thereafter renewed every 2 - 3 days. Confluent flasks were trypsinised one into two at first, and later one into four or more. Recently, a versene-trypsin was used for routine passage (0,125% trypsin, 0,05% EDTA and 0,05% glucose in PBS). Growth medium, as defined above, was used consistently.

Chromosome Preparation

The medium of well-growing, nearly confluent, hepatoma cell cultures was changed 24 hours before chromosome preparation. The cultures were incubated for 24 hours at 37°C in normal growth medium to which colchicine (BDH) had been added to a final concentration of 0,5 µg/ml. This medium was exchanged for 0,5% sodium citrate. After 20 minutes at 37°C the cells detached from the surface and were transferred to 10-mi conical centrifuge tubes. To improve compaction of pelleted material 1,5 ml of bovine serum was added to each 8,5 ml of suspension. After centrifugation for 5 minutes at 1500 g the pellet was resuspended in freshly prepared fixative, methanol : acetic acid (3 : 1), and the suspension kept at room temperature for 20 minutes. After centrifugation for 5 minutes at 1500 g the pellet was resuspended in fixative and stored at 4°C overnight. The final pellet was resuspended in a little cold fixative for spreading the chromosome plates. A few drops of the suspension were placed onto cold coverslips, these were flame-dried, stained with Giemsa at pH 6,8 and mounted in DePeX. The slides were examined under oil for karyotyping, and 100 complete chromosome spreads were photographed on Ilford Pan F film.

Enzyme studies: 700 mg of hepatoma cells were scraped from the flasks, washed once with PBS and assayed for glucose-6-phosphate dehydrogenase, lactic dehydrogenase, hexokinase and pyruvate kinase isozymes.⁹

AFP measurements: Culture medium which had been in contact with cells for 7 days was clarified by centrifugation (3 000 g/30 min). The supernatant was assayed by radio-immunoassay.⁷ Growth medium which had not been in contact with cells was used as a control.

Electron microscopy: Confluent cell cultures were washed four times with PBS and prefixed with 3% glutaraldehyde in PBS (pH 7,2) for 1 hour at room temperature. The cells were scraped from the flasks and pelleted. The pellet was postfixed in 1% osmium tetroxide, dehydrated in ethanol, embedded in Epon, and sectioned. After double staining in uranyl acetate and lead citrate, sections were viewed in a Philips EM 300 electron microscope.

RESULTS

Histological examination of postmortem liver tissue showed that there was cirrhosis and malignant neoplasia with features indistinguishable from hepatoma, and the material used for tissue culture was considered to be malignant. Ultrastructural examination of the original tumour tissue showed cells with large nuclei, lysosome-like structures and no discernible virus particles.

Of the original 14 flasks, one had about 20 fragments attached to the surface within 2 days, and two other flasks had 2 -3 attached cell clumps. Each fragment consisted of 20 -100 cells. All three cultures had been derived from the secondary tumour mass in the liver and each had been trypsinised for 5 minutes. Within 5 days the attached clumps were surrounded by two-dimensional areas of epithelial cell outgrowth and an occasional mitosis was seen. The two flasks with few attached pieces were maintained for 6 weeks when cell degeneration occurred and the flasks were discarded. Cell division continued in the remaining flask, but death of cells was pronounced, and no over-all increase in numbers of cells occurred for about 5 weeks. Thereafter the death rate declined and the cells increased in number. A major problem at this stage was the presence of healthy, vigorously growing fibroblasts which were initially removed mechanically by scraping around the epithelial cell islands with a 16-gauge needle. Later, differential trypsinisation with mechanical scraping reduced the population of fibroblasts in the culture. The tumour cells were first subcultured after 4 months in vitro. Initially it was difficult to suspend the cells with trypsin but this improved with higher passages. From the 5th passage versusen-trypsin was used because this gave a more uniform cell suspension and a higher plating efficiency. Since the 4th passage the cultures have been completely free of contaminating fibroblasts. The tumour cells were considered adapted to in vitro conditions after 1 year (8th - 9th passage) but were regarded as a cell line only after 18 months in culture when it became possible to store the cells at -70°C in a freezing mixture containing 40% fetal bovine serum, 50% medium and 10% glycero1 and to re-initiate growth after thawing. At present (40th passage) the cultures can be passaged at 1:6 dilution, the plating efficiency is 40 - 50% and the cell doubling time is 35 - 40 hours. The cells (Fig. 1) are polygonal in shape with well-defined borders. Confluent cultures or confluent areas within cultures have a greater proportion of smaller cells compared with the edges of growing islands, and in these cells the cytoplasm is more granular. Many of the cells are binucleate, which is a property of hepatocytes in culture.¹⁰ The growth pattern in vitro is typical for epithelial cells: islands of outgrowth occur as the cells multiply in sparse culture and trypsinisation produces large clusters of tightly packed cells. Trypsin plus versene is required for monodisperse suspensions. The morphology of the cells has not altered since initial isolation. Cell division does not stop in confluent cultures and although the cells do not pile up, large numbers detach from the surface. Over 90% of these cells, harvested from the supernatant, are dead, as judged by trypan blue uptake.

The karyology of the cells is male and human (Fig. 2).
The numerical chromosome analysis of 100 complete and well-spread metaphase plates of the hepatoma cells showed a distribution of 48 - 61 with a mean number of 56. A feature is that over 90% of all plates have chromosomes missing in the D group and over 70% have lost more than one chromosome from the G group. Apart from several other marker chromosomes, apparent D/G fusions were identified in almost all spreads.

**Enzyme Studies**

The enzymes conformed to the human pattern, and the cells contain the type A glucose-6-phosphate dehydrogenase.

**AFP Determinations**

Extremely low levels were detected — 3 - 4 ng/ml. This was approximately the same as the levels measured in the control medium — 2.5 - 3 ng/ml. Since the growth medium contains fetal bovine serum, we are at present attempting to adapt cultures to growth on adult bovine serum.

**Electron Microscopy**

Fig. 3 shows that ultrastructurally the cells have a dense cytoplasmic matrix with few elements of the endoplasmic reticulum, abundant polysomes and mitochondria with a dense matrix. Lysosome-like structures containing membrane fragments and other electron-dense material are common. Sparsely distributed glycogen granules are evident and some areas of the cell surface are particularly rich in microvilli. Bundles of filaments are occasionally found in the cytoplasmic matrix. The nuclei are characterised by the absence of condensed chromatin, either marginal or in clumps dispersed in the nucleoplasm. The nuclear envelope often shows indentations, occasionally to a marked degree. Most cells have more than one nucleolus...
which invariably consists of the nucleolomata only, with no evidence of the pars amorphus. Virus particles were not detected by electron microscopy.

**DISCUSSION**

The establishment of a new cell line from a human tumour is an infrequent event, and in describing the characteristics of a cell line, the following possibilities should be discussed:

1. Did the cell line arise as a consequence of cross-contamination from other existing cell lines?
2. Did the cell line develop from a single cell which mutated after the material had been prepared for tissue culture, or from normal cells which may have been present at the time of explant?
3. Do the cells in *vitro* retain many characteristics of the cells in the original tumour, and as a corollary;  
4. Are the cells malignant?

We shall deal with each of these points separately.

1. Some 'new' cell lines have resulted from contamination by other established cultures, especially HeLa. Apart from the fact that we had no HeLa cultures in the laboratory during the period that the hepatoma cell line was established, we took great care in handling the original culture flask. The cells grow out from the other three specimens.

2. A cell mutating in *vitro* may, after weeks or months in culture, give rise to a clone, the cells of which grow indefinitely. The cells described here did not develop from an isolated group of cells — the primary culture contained about 20 islands of cells and the in *vitro* properties reported here relate to cultures derived from outgrowth from these multifocal areas in the primary culture. We have ruled out the possibility that we are dealing with normal liver cells which have 'adapted' to in *vitro* growth because the material removed for tissue culture was histologically described as malignant and many attempts to establish normal human liver cells from both adult and fetal tissue in our laboratory using identical techniques resulted only in sparse cultures of epithelial cells which did not divide, could not be subcultivated and were rapidly overgrown by fibroblasts.

3. The morphology of the cells by light microscopy and the ultrastructural features indicate a resemblance to liver cells in most respects. Electron microscopy of the original tumour showed cells with lysosome-like bodies and no discernible viral structures. These characteristics have been maintained in *vitro*. The apparent lack of AFP production by the hepatoma cells may have resulted from adaptations to in *vitro* growth and we did not test early passage supernatants. The genetic stability of cell lines in *vitro* is unknown and apart from periodic karyotyping no techniques are currently available for measuring stable over-all genetic function. Chromosome analysis has shown that the cells are human and male. Heteroploidy is commonly found in established cell lines and the hepatoma cells are remarkable in that the large number of D/G translocations are a consistent feature together with other marker chromosomes (Fig. 2).

The question as to whether or not the hepatoma cell line is truly malignant is more difficult to answer. The conclusive test for malignancy is that cells, when inoculated into the same organism, produce tumours. Heterotransplantability is not an absolute definition and while we have inoculated the cells into immunosuppressed hamsters, to date no tumours have developed. The AFP-producing epithelial cell line did not produce tumours in hamsters although the fibroblastic cell line did. However, the hepatoma cells described in this report do grow in soft agar (R. Saunders, personal communication) and this ability is commonly regarded as a definitive test for malignancy.

The over-all evidence supports the claim that the cell line described here was derived from male, human, malignant liver tissue. This claim is further strengthened by the recent finding that these cells produce hepatitis B surface antigen-reactive material.

Previous to the hepatoma described here, we prepared four other liver tumour specimens over a period of 6 months for growth in *vitro*. One of these was heavily contaminated with micro-organisms, while only fibroblasts grew out from the other three specimens.

Since it is advisable to compare this cell line with others which may subsequently become established from primary liver carcinomas, we have designated this one PLC/PRF/5.

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**REFERENCES**