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Abstract

Cancer cells obtained from human hepatocellular carcinoma nodules were subjected to primary culture, and a hepatoma cell line was established. The cell clumps obtained by needle puncture were plated directly in plastic tissue culture flasks without any cell dissociation procedures. Cell clusters became attached to flasks in 24 h with an efficiency of about 90%. No fibroblast outgrowth was observed. Primary cultured cells were composed of polygonally shaped epithelial cells with dense cytoplasm and one or more large nuclei. They excreted plasma protein biosynthetic markers of hepatocytes into the culture medium. Plasma protein synthesis of primary cultured hepatoma cells decreased as the age of the primary cultures increased. Cells seeded in September 1980 started to grow continuously after 5 months of cultivation. A new hepatocellular carcinoma cell line (designated as KG55T) was established from these growing cells. KG55T cells have been subcultured for more than 20 passages and form a monolayer of polygonal epithelial cells which pile up after they reach confluence. The cells had a doubling time of 50-60 h and a plating efficiency of 60-65%. Albumin, alpha 1-antitrypsin and alpha 2-macroglobulin syntheses and tyrosine aminotransferase activity were detected. At the 10th passage, KG55T cells were pseudotriploid (mode, 69), and 8q+ and 15q+ translocations were distinctive of this cell line. The morphological characteristics and the capacity for plasma protein synthesis of the primary cultured hepatoma cells and cells of the established hepatoma cell line were compared.

KEYWORDS: primary culture, human hepatoma cell line, plasma protein

PRIMARY CULTURED CELLS AND AN ESTABLISHED CELL LINE OF HUMAN HEPATOCELLULAR CARCINOMAS

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Abstract. Cancer cells obtained from human hepatocellular carcinoma nodules were subjected to primary culture, and a hepatoma cell line was established. The cell clumps obtained by needle puncture were plated directly in plastic tissue culture flasks without any cell dissociation procedures. Cell clusters became attached to flasks in 24 h with an efficiency of about 90%. No fibroblast outgrowth was observed. Primary cultured cells were composed of polygonally shaped epithelial cells with dense cytoplasm and one or more large nuclei. They excreted plasma protein biosynthetic markers of hepatocytes into the culture medium. Plasma protein synthesis of primary cultured hepatoma cells decreased as the age of the primary cultures increased. Cells seeded in September 1980 started to grow continuously after 5 months of cultivation. A new hepatocellular carcinoma cell line (designated as KG55T) was established from these growing cells. KG55T cells have been subcultured for more than 20 passages and form a monolayer of polygonal epithelial cells which pile up after they reach confluence. The cells had a doubling time of 50-60 h and a plating efficiency of 60-65%. Albumin, $a_1$-antitrypsin and $a_2$-macroglobulin syntheses and tyrosine aminotransferase activity were detected. At the 10th passage, KG55T cells were pseudotriploid (mode, 69), and 8q+ and 15q+ translocations were distinctive of this cell line. The morphological characteristics and the capacity for plasma protein synthesis of the primary cultured hepatoma cells and cells of the established hepatoma cell line were compared.

Key words: primary culture, human hepatoma cell line, plasma protein.

Several laboratories have succeeded in establishing human hepatoma cell lines with cytological, biochemical and genetic characteristics of liver parenchymal cells (1-9). It has been shown that these cell lines have normal liver parenchymal cell functions such as biosynthesis and secretion of plasma proteins (4, 5, 10). Three cell lines have been described as retaining the ability to produce hepatitis B surface antigen (HBsAg) (1, 4, 11-13). Integration of hepatitis B virus DNA into the cellular DNA was demonstrated in HBsAg producing cell lines (14-17). Such human hepatoma cell lines with differentiated liver functions have broadened the scope of primary liver cancer research.

However, it is laborious to prepare an appropriate number of hepatoma cells in primary culture since associated with primary culture are such difficulties as
cell dissociation, low attachment efficiency and fibroblast outgrowth. No experimental study using primary cultured hepatoma cells has been reported, and the establishment of a human hepatocellular carcinoma cell line is still a chance event (18).

Cancer cells in clumps obtained by needle puncture of hepatocellular carcinoma nodules under a peritoneoscope were viable and explanted without any cell dissociation procedures. We used the cells as such for primary culture and establishment of a hepatoma cell line.

In this paper, the characteristics and capacity for producing plasma proteins of primary cultured hepatoma cells and cells of an established hepatoma cell line (KG55T) are described.

MATERIALS AND METHODS

Cell preparation. Cancer cells were obtained from three cirrhotic patients with hepatocellular carcinoma. The first patient was a 53-year-old male who was negative for serum HBsAg and positive for hepatitis B surface antibody (anti-HBs) and had 65 μg/ml α-fetoprotein (AFP). The second patient was a 55-year-old male having neither serum HBsAg nor anti-HBs, and having 39.0 ng/ml AFP. The third patient was a 40-year-old male who was positive serum for HBsAg and negative for anti-HBs and had 930 μg/ml AFP.

Cancer cell clumps, which flowed spontaneously from the cancer nodules through a Vim-Silverman's needle (2.5 mm outside diameter), were obtained using a peritoneoscope (19). The viability of the cells was determined by the trypan blue dye exclusion test. The material was divided into two portions, one for histological examination and the other for cell culture.

Primary culture. Cancer cell clumps were transferred into a sterile tube containing 10 ml of chilled Ham's F-12 medium (Grand Island Biological, Co., Grand Island, N.Y.) supplemented with 5% fetal bovine serum, 2.4 μg/ml dexamethasone (Sigma Chemical Co., St. Louis, Mo.) and 20 μg/ml hydrocortisone (20). The tubes were agitated gently, and 5 ml aliquots, of free cells and several cell clumps, containing 1×10⁶ cells were plated directly in 25-cm² plastic tissue culture flasks (Corning Glass, Corning, N.Y.) without dissociating the cells. Growth medium was replaced with 5 ml of fresh medium on the next day and every 2 to 3 days thereafter. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Four weeks after plating, Ham's F-12 medium supplemented with 10% fetal bovine serum was used for further cultures.

Subculture and establishment of hepatocellular carcinoma cell line. Growing cells of the primary culture derived from the carcinoma of the first patient were subcultured after dispersing the cells with 0.05% trypsin in calcium- and magnesium-free Hanks' balanced salt solution. Subsequently, versene-trypsin (0.05% trypsin, 0.02% EDTA in calcium- and magnesium-free Hanks' balanced salt solution) was used for routine passages of confluent cultures. Cells of each passage were stored in liquid nitrogen with 10% dimethylsulfoxide in complete media.

Morphological studies. Cancer cell clumps were fixed in Bouin's solution for light microscopy and fixed with 1% glutaraldehyde by puncture perfusion for scanning electron microscopy (21). A phase-contrast microscopic study was conducted on monolayer cultures.

Detection of human plasma proteins in tissue culture medium. Proteins secreted into culture medium were analyzed at 30, 60, 90, 120, and 150 days of primary culture by the Ouchterlony double-diffusion technique (22) using commercially available antisera (Behringwerke AG, Mar-
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burg, W. Germany) to the major human plasma proteins. The culture medium of KG55T cells (4 \times 10^4 cells/25-sq cm flask) was also examined. Growth medium in which the cells were cultured for 3 days was concentrated twentyfold with a Minicon-B 15 concentrator (Amicon, Lexington, Mass.) prior to immunodiffusion. Fresh medium concentrated in the same manner was used as a control. Growth medium was also tested for AFP by enzyme-immunoassay using an AFP-EIA Diagnostic Kit (Abbott Lab., Ltd., Tokyo, Japan) and for HBsAg by reversed passive hemagglutination.

Growth kinetics and plating efficiency. The population doubling time of KG55T cells was determined during the logarithmic growth phase. Cells were seeded at an initial density of 2 \times 10^5 cells/well (17 mm in diameter) in a 3008 type Multiwell Tissue Culture Plate (Falcon, Cockeysville). Cells dispersed at 24-h intervals for up to 20 days were counted in duplicate with a hemocytometer. The plating efficiency was determined by calculating the ratio of the number of colonies formed to 95 %, to compensate for doubling, of the actual number of cells seeded in a 25-sq cm culture flask.

Chromosomal analysis. Chromosomal analysis of KG55T cells was performed at the 10th passage. Subconfluent cells were incubated with medium containing 1.0 μg/ml of colchicine for 2 h at 37 °C. The cells were dispersed with versene-trypsin, and the cell suspension was made hypotonic with 0.075 M KCl for 25 min. They were fixed in Carnoy's solution (methanol : acetic acid = 3 : 1, v/v) and subsequently air-dried on clean slides. Giemsa banding of the chromosomes was carried out using the method of Seabright (23) with minor modifications in the cell treatment with 0.025 % trypsin for 15 to 20 sec and the staining with Giemsa reagent. Classification of the chromosomes was based on the International System for Human Cytogenetic Nomenclature (1978) (24).

Histochemical enzyme analysis. KG55T cells were examined for their tyrosine aminotransferase activity by a histochemical staining technique based on the reduction of tetrazolium salts as described by Thompson and Tomkins (25). The substrate monoiototyrosine was omitted in the negative control.

Tumorigenicity. KG55T cells (5 \times 10^4-1 \times 10^7 cells) were subcutaneously injected into 3-week-old nude mice (BALB/c, nu/nu).

RESULTS

Morphology and tissue culture. No significant complications of the laparoscopy and liver biopsy occurred in this study. The diagnosis, based on histology, was of hepatocellular carcinoma for all three patients. Cells in the cancer tissue were loosely connected to each other in clumps which were surrounded by a single layer of endothelial cells (Figs. 1, 2). The trypan blue exclusion test showed that over 90 % of the cells were viable. Cells obtained from all patients became attached to the flasks in 24 h with an efficiency of about 90 %. Cell colonies of primary cultures were maintained in a viable state without any cell proliferation (Fig. 3). Cancer cells in the primary culture were polygonal in shape with dense cytoplasm and one or more large nuclei. The cells did not change morphologically over a period of 5 months.

Cells obtained from the first patient and seeded in September 1980 started to grow continuously after 5 months of cultivation. The growing cells were subcultured after 9 months for the first time and have been passaged more than 20
Fig. 1. Photomicrograph of cancer cell clumps, i.e., hepatocellular carcinoma cells surrounded by endothelial cells, obtained by needle biopsy. Hematoxylin and Eosin, ×125.

Fig. 2. Scanning electron micrograph of cancer cell clumps. C: cancer cells. E: endothelial cells. ×400.

Fig. 3. Polygonal hepatocellular carcinoma cells with dense cytoplasm and one or more large nuclei, in primary culture. Fibroblast outgrowth is not seen. Phase-contrast microscopy. ×125.

Fig. 4. KG55T cells with clear cytoplasm and small granules at the 10th passage. Phase-contrast microscopy. ×125.

Fig. 5. Immunoprecipitates of the Ouchterlony double immunodiffusion. Central wells: antisera to: (1) albumin, (11) α₁-antitrypsin and (111) α₂-macroglobulin. Surrounding wells: Concentrated media of: (1) 30 days, (2) 60 days, (3) 90 days, (4) 150 days of primary culture, (KG) KG55T cells and (C) control.

times. Cells of this cell line (KG55T) have a polygonal shape with clear cytoplasm and small granules, characteristic of epithelial cells. They form a monolayer and pile up after reaching confluence. Mitotic cells are also to be seen (Fig. 4). Cells from the second and third patients have been maintained in primary culture for 4 and 3 months, respectively.

*Human plasma proteins in tissue culture medium.* Albumin, α₁-acid glycoprotein, α₁-
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Table 1. Identification of plasma proteins in tissue culture media of primary cultured cells and KG55T cells by the Ouchterlony double-diffusion technique.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primary cultured cells from patient 1 (30) (60) (90) (120) (150)</th>
<th>Primary cultured cells from KG55T (7) (30) (60) (90)</th>
<th>Primary cultured cells from patient 2 (7) (30) (60) (90)</th>
<th>Primary cultured cells from patient 3 (7) (30) (60) (90)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α1-Fetoprotein</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α1-Acid glycoprotein</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>α1-Antitrypsin</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>α2-HS-glycoprotein</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>α2-Macroglobulin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-Lipoprotein</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Transferrin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Complement C3</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Complement C4</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>C3 activator</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Secretory component</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>HBsAg</td>
<td>-</td>
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</tbody>
</table>

Numbers in parentheses indicate days of culture.

antitrypsin, &alpha;2-macroglobulin, transferrin, fibrinogen, and the complement components C3 and C4 were detected in the medium of cells from the first patient throughout the primary culture, and &alpha;2-HS-glycoprotein and ceruloplasmin were detected until the 90th day (Table 1). AFP, &beta;-lipoprotein, C3 activator, the sec-

Fig. 6. Growth curve of KG55T cells at the 12th passage.
retry component, and HBsAg were not detected by the method employed. Cells from the other two patients also excreted major plasma proteins, though several became undetectable later in the primary culture (Table 1). In the culture medium of KG55T cells, albumin, α₁-antitrypsin and α₂-macroglobulin were detected by the Ouchterlony immunodiffusion method (Fig. 5). They continue to secrete these proteins at the time of this writing (23rd passage).

![Graph showing chromosome distribution of KG55T cells at the 10th passage.](image)

Fig. 7. Chromosome distribution of KG55T cells at the 10th passage.

![Karyotype of KG55T cells at the 10th passage.](image)

Fig. 8. G-banded karyotype of KG55T cells at the 10th passage. Formula: 73, XXY, +1, +2, +3, +6, +7, +8q+, +9, +9p−, +10p−, +11, +12, +12, −13, +13q−, +15q+, +15q+, +M1, +M2, +M2, +18, +18, +19, +19, +20, +20, −22, +M3, +M3, +M4, +M5.
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Fig. 9. Tyrosine aminotransferase staining of KG55T cells at the 10th passage. Left: Positive staining. Right: Negative control. × 125.

**Growth curve and plating efficiency.** The growth characteristics of KG55T cells were studied at the 12th passage. The cells grew exponentially 24 h after seeding, and the growth curve reached a plateau at about $1.5 \times 10^4$ cells/17-mm-dia well (Fig. 6). The population doubling time was 50-60 h and the plating efficiency was 60-65 %. The cells stored in liquid nitrogen and then thawed exhibited the same morphology and growth pattern as before freezing.

**Chromosomal analysis.** Chromosome counts of KG55T cells were based on 50 metaphase spreads performed at the 10th passage and revealed a pseudotriploid chromosome pattern. The chromosome number ranged from 51 to 75 with a mode of 69 (Fig. 7). Studies of the banded karyotypes revealed evidence of structural as well as numerical aberrations in the pseudotriploid cells (Fig. 8). Several types of translocation were distinctive of this cell line, especially 8q+ and 15q+. The male sex chromosome was observed.

**Histochemical enzyme analysis.** KG55T cells at the 10th passage exhibited a definite color reaction to tyrosine aminotransferase staining (Fig. 9).

**Tumorigenicity.** No tumors developed at the site of inoculation during a 5-month observation period.

**DISCUSSION**

The present study demonstrated plasma protein synthesis of primary cultured hepatoma cells and establishment of a hepatoma cell line (KG55T). Previously reported hepatoma cell lines were derived from various cell sources (1, 2, 4-6, 9). Major problems, however, in the previous studies included low attachment efficiency and fibroblast outgrowth in primary culture. We also have attempted to cultivate human hepatocellular carcinoma cells derived from 5 operative and 2 autopsy specimens, but none of them could be maintained as a long-term culture.

The cancer tissue we obtained by needle puncture of cancer nodules consisted of free cancer cells and cells in clumps surrounded by a single layer of endothelial
cells and was probably the same tissue described by Hamperl (26) as "cancer milk" (loosely connected cancer cells). The cells we obtained were viable and became attached to tissue culture flasks efficiently. Neither chemical nor enzymatic dissociation procedures were necessary for seeding, and fibroblast outgrowth was not found throughout the culture. Enough cancer cells \(10^2-10^4\) cells for the primary culture were obtainable in each case. These cells provided a good experimental model for the investigation of human hepatocellular carcinoma and were a good source for the establishment of a hepatocellular carcinoma cell line.

Recently, in order to overcome the problem of cell attachment in primary culture, insoluble fibers called biomatrix (27, 28), which contain various collagens and several glycoproteins such as fibronectin (29), have been utilized. Rat hepatocytes cultured on rat liver biomatrix have shown increased attachment and survival efficiencies, duration of the cultures, and retention of some liver specific functions (27). Human hepatocellular carcinoma cells plated on an extracellular matrix produced by endothelial cells had a high growth rate and a low serum requirement, and formed a cell monolayer composed of firmly attached, highly flattened and closely packed epithelioid cells (30). Thus, matrices produced by cultured cells or extracted from the rat liver can provide the proper substratum for maintaining isolated hepatocytes for several months in culture (28). With this fact in mind, the endothelial cells which surrounded the cancer cell clumps may have contributed to the high attachment efficiency and the long-term maintenance of the hepatoma cells without outgrowth. The role of such endothelial cells in the long-term maintenance of primary cultures needs to be investigated further.

A new cell line (KG55T) was established from the growing cells of the primary culture derived from hepatocellular carcinoma cells from the first patient using a routine technique for subculture. The plating efficiency was high from the first passage. The cells have a pavement-like arrangement and form multilayers. Karyological analysis showed a wide distribution of chromosome number with a pseudotriploid mode differing from the normal diploid number. The male sex chromosome was observed. The cells retained liver specific functions, as demonstrated by their plasma protein synthesis and tyrosine aminotransferase activity. These cells grow sufficiently well to yield reproducible populations for experimental use.

Plasma protein synthesis has been investigated using perfused liver (31, 32), liver slices (33), isolated liver cell suspensions (34, 35), primary cultures of isolated hepatocytes(36, 37), and hepatoma-derived cell lines (38-40) in animal experiments. Isolated hepatocytes or perfused liver have the problem of being too short-lived, and hepatocyte functions have been retained for only a few days. The development of a model system for studying the regulation and differentiation of plasma protein synthesis has been done with primary cultures of chick embryo liver cells which kept the functions for several days (41-43). Investigations of plasma protein synthesis in humans have been reported using hepatoma cell lines (10) and clonal
cell strains of normal liver (44). However, no primary culture system for the investigation of plasma protein synthesis in human hepatoma cells has been described. The primary cultured hepatoma cells derived from the first patient in this study continued to excrete a variety of plasma proteins for 5 months, though the secretion of α₅-HS-glycoprotein and ceruloplasmin could be detected only for 90 days. There was no detectable AFP from the initiation of primary culture, though it was high in the patient's serum. In the case of the first patient and the other two, the secretion of plasma proteins decreased as the age of the primary cultures increased.

In the culture medium of KG55T cells, α₅-acid glycoprotein, transferrin, fibrinogen, the complement components C3 and C4 were not detected with the Ouchterlony immunodiffusion method. Plasma protein production of KG55T cells was initially less than that of the primary cultured cells, but there was no decrease in the plasma protein synthesis of KG55T cells according to the number of subcultures. The identification of other plasma proteins secreted by KG55T cells would be of value to further research.

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