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Induction of Plasma Protein Secretion in a Newly Established Human Hepatoma Cell Line

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To study the expression and the regulation of hepatocyte markers, we have undertaken to establish human hepatoma cell lines of various phenotypes. We now report the establishment of a new human hepatoma cell line, HA22T/VGH. This cell line has many of the properties of human hepatocellular carcinoma. Only 5 of 15 plasma proteins investigated were detected in the medium of a 10-day-old HA22T/VGH culture. However, when the HA22T/VGH cells and a clonal derivative, C5, were cultured in an aggregated form, all 15 plasma proteins were found in the culture medium. These results indicate that hepatoma cell lines with different phenotypes can be established, and they provide a good experimental framework to investigate differentiation of human hepatocytes.

The control of cell differentiation remains one of the most challenging problems in biology. The differentiation of human hepatocytes seems to be particularly interesting since varieties of plasma protein markers have been well characterized. Furthermore, hepatocellular carcinoma, a hepatocyte malignancy, is a common neoplasm and a primary cause of cancer death in many Asian and African countries.

An experimental model for studying the differentiation of human hepatocytes is still lacking at present. Recently, three human hepatoma cell lines, Hep G2 and Hep 3B, which secrete 16 plasma proteins, and PLC/PRF/5, which secretes 7 of these proteins, have been established (1, 2, 12). These results suggests a possible approach for studying human hepatocyte differentiation, by establishing human hepatoma cell lines with various phenotypes and then studying the expression and regulation of hepatocyte markers, as well as the induction of hepatocyte differentiation in vitro.

We have established a new human hepatoma cell line (HA22T/VGH). This cell line has the characteristics of human hepatocellular carcinoma and secretes five plasma proteins only. However, after cell aggregation in vitro, HA22T/VGH and its clonal derivative, C5, can be induced to secrete all 15 of the plasma proteins we investigated. Therefore, these studies provide a good experimental model for investigating the gene regulation and differentiation of human hepatocytes.

MATERIALS AND METHODS

Establishment of the HA22T/VGH cell line. The HA22T/VGH cell line was established from a surgical specimen of hepatocellular carcinoma obtained from a 56-year-old Chinese male. The specimen was minced into approximately 1-mm^3 pieces and seeded on a 25-cm^2 flask (Falcon Plastics, Oxnard, Calif.) precoated with fetal bovine serum (FBS). Cells were incubated at 37°C in an atmosphere of 5% CO_2 in air and in Dulbecco modified Eagle medium (DMEM) which was supplemented with 10% FBS, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. Two months later, polygonal cells surrounded by spindle-shaped fibroblasts were observed. The culture was then differentially trypsinized according to the techniques of Alexander et al. (2), and pure polygonal cells without spindle-shaped fibroblasts were obtained after three passages.

Cell lines and cell cultures. Cell lines Hep 3B, Hep G2, and SK-Hep-1 were provided by D. A. Aden, B. B. Knowles, and J. Fogh (1, 2, 7). Cells were grown in DMEM supplemented with 10% FBS, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml.

Chromosome analysis. Cells of HA22T/VGH at passage 10 were examined karyotypically. Two hours before harvesting, 0.01 ml of colcemid (demecolcine, Ciba-Geigy; 10 μg/ml) per ml of the medium was added to the culture flask, in which a good number of mitotic cells could be seen. Cells were collected after trypsinization and treatment with hypotonic solution (0.07 M KCl) at room temperature. Several drops of freshly prepared fixative (methanol-acetic acid, 3:1) were added and mixed thoroughly with the cell-containing hypotonic solution to prevent cell clumping during fixation. The cell pellet was washed three times with 3 ml of fixative. After storage overnight or longer in the

refrigerator, the cell suspension was spread on a cold, wet glass slide. Giemsa staining by both conventional and C-banding (15) methods was applied to the slides after air drying. A total of 20 to 50 well-spread metaphases were analyzed.

Growth in methylcellulose suspension. The growth of HA22T/VGH in methylcellulose was performed as described previously (14). Briefly, a feeder layer consisting of 3 ml of 0.9% agar (Bacto-Agar; Difco Laboratories, Detroit, Mich.) in DMEM, 10% FBS, and antibiotics was allowed to solidify in plastic petri dishes (60 by 15 mm). DMEM culture medium (4 ml), containing 1.3% methylcellulose (4,000 centipoises) and 10^5, 10^4, 10^3, or 10^2 cells, was poured onto the agar layer, and four replicate dishes were prepared for each dilution. Methylcellulose medium (4 ml) was then added to each culture dish every 10 days. The number of visible colonies was scored 3 weeks after the initial plating.

Determination of tumorigenicity in nude mice. Six-week-old athymic nude mice were purchased from the Animal Production Section, Institute of Preventive Medicine, Taipei, Taiwan, Republic of China. Each nude mouse was injected subcutaneously with 10^7 or 10^6 cells. After the tumors developed in the nude mice, they were excised and examined histologically.

Cloning of HA22T/VGH cells. HA22T/VGH monolayer cells were trypsinized from flasks, and the cell number was counted. Cells were then suspended into DMEM culture medium to a final concentration of 0.4 cells per 0.1 ml. To each well of a 96-well culture plate (Falcon Plastics) 0.1 ml of the cell suspension was added. The clonal growth derived from a single cell was examined under a microscope every day.

Cell aggregation. Cell aggregation was performed in bacteriological petri dishes (Chi-Hsin Co., Taipei, Taiwan, Republic of China) on which most of the cells did not attach. Cells in monolayer culture were detached and inoculated onto the bacteriological petri dishes (2×10^6 cells in 10 ml of medium). They were incubated at 37°C and in 5% CO_2 in air. The HA22T/VGH cells aggregated spontaneously. The culture media were collected 10 days later. The cell aggregates were trypsinized, and the cell numbers were counted.

Assays. The complement fixation (CF) tests for the plasma proteins were performed by the standard CF method (4). The antisera against each plasma protein were purchased from Calbiochem-Behring Co., San Diego, Calif. Tyrosine aminotransferase (EC 2.6.1.5) and alanine aminotransferase (EC 2.6.1.2) activities were determined as described by Granner et al. (8) and Henry et al. (9), respectively, on cell extract prepared as described by Johnson and Kenney (10). γ-Glutamyltransferase (EC 2.3.2.2.) activity of the cell-free extract was determined as described by Cameron et al. (3). One unit of enzyme activity is the quantity which catalyzes the formation of 1 μmol of product per min at 37°C.

The hepatitis B virus surface antigen was detected with a solid-phase radioimmunoassay kit (Connaught

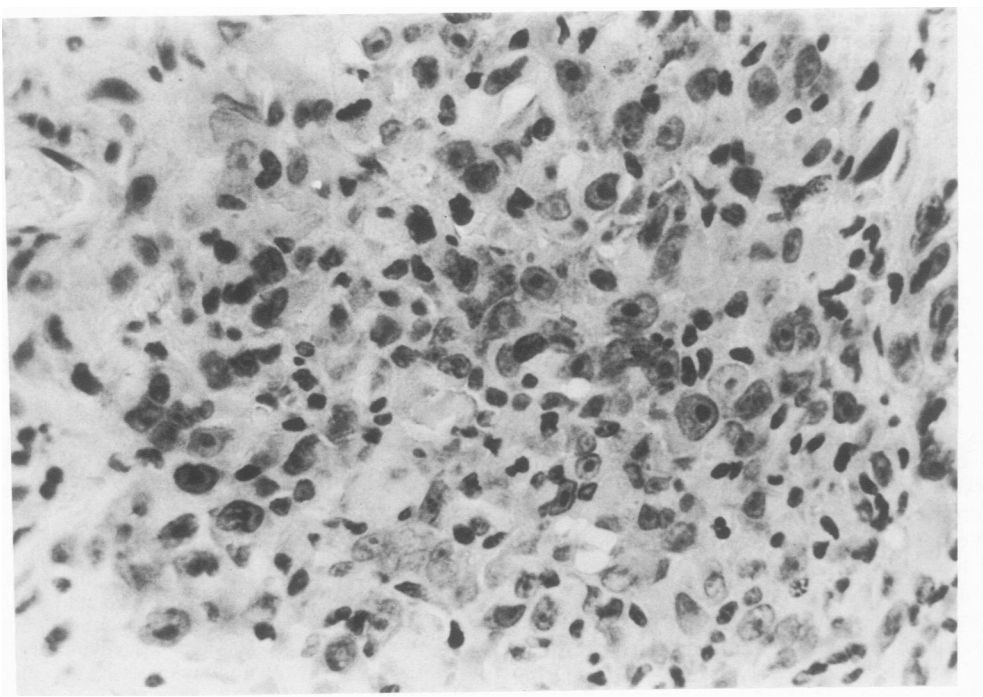

FIG. 1. Histopathological picture of the tumor from a nude mouse injected with HA22T/VGH cells. HA22T/VGH cells were harvested and injected subcutaneously at a dose of 10^7 cells into nude mice. Tumors were observed 2 weeks after injection. They were excised, fixed in Formalin, and embedded in paraffin. This section was stained with hematoxylin-eosin and examined under a microscope. The tumor cells are somewhat distorted in shape but still can be identified as hepatoma cells. Magnification, ×400.

Labs, Willowdale, Ontario, Canada). The α-fetoprotein was also analyzed with a radioimmunoassay test kit (Serono Diagnostics, Coinsins, Switzerland).

RESULTS

General properties of HA22T/VGH. The HA22T/VGH cell line has now been passaged over 100 times and stored in liquid nitrogen at various passages. This cell line has an abnormal chromosome number with a mode of 73. Studies on the properties of anchorage independence showed that an unusually high percentage (40%) of cells formed colonies on methylcellulose-soft agar. The HA22T/VGH cells also induced tumors in nude mice at a dosage of 10^6 cells. Histologically, the tumors of nude mice could be identified as hepatocellular carcinoma (Fig. 1). Analyses on the hepatocyte-associated enzymes indicated that the HA22T/VGH cells contained alanine aminotransferase, tyrosine aminotransferase, and γ-glutamyltransferase. By comparison with the Hep 3B cell line, however, the HA22T/VGH cells had a much lower amount of alanine aminotransferase and γ-glutamyltransferase than did the Hep 3B cells (Table 1). The HA22T/VGH cells did not secrete hepatitis B surface antigen and α-fetoprotein, as detected by radioimmunoassay.

Plasma protein secretions of human hepatoma cell lines. Plasma protein synthesis is a specialized function of hepatocytes (11). The synthesis and secretion of plasma proteins of HA22T/VGH, Hep G2, Hep 3B, and SK-Hep-1 were tested by double immunodiffusion with commercially available antisera to the major human plasma proteins as described previously by Knowles et al. (12). Among the 15 plasma proteins tested (Table 2), Hep G2 secreted 14 plasma proteins with the exception of G_c-globulin; Hep 3B produced 14 plasma proteins with the exception of the fourth complement component (C4). Under the same conditions, HA22T/VGH secreted only ceruloplasmin and C3 activator; SK-Hep-1 cells did not secrete any plasma protein. Further studies by the more sensitive and quantitative CF technique showed that Hep G2 also secreted G_c-globulin with a titer of 32. In addition to ceruloplasmin and C3 activator detected by the double immunodiffusion test, HA22T/VGH was found to secrete G_c-globulin, C3, and a small amount of C4. None of the plasma proteins was detected in the supernatant fluid of the SK-Hep-1 cell line by the CF test (Table 2).

TABLE 1. Activities of hepatocyte-associated enzymes from two hepatoma cells lines

Cell line	Enzyme activity (mU/mg)[a]		
	AAT	TAT	γ-GT
HA22T/VGH	8.60	3.01	5.5
Hep 3B	30.27	2.92	64.8

[a] Abbreviations: AAT, alanine aminotransferase; TAT, tyrosine aminotransferase; γ-GT, γ-glutamyltransferase.

Plasma protein secretions of aggregated HA22T/VGH cell line. Cell contact and cell interaction seem to play an important role in cell differentiation and embryonic development (6, 13). To increase cell contacts, HA22T/VGH cells were grown as aggregates in bacteriological petri dishes. The cells spontaneously formed smooth, round, and sometimes chainlike aggregates. These aggregated cells initially had a doubling time similar to monolayer cells. However, these cells stopped growing at a lower density than did the monolayer cells. Interestingly, histological studies of the HA22T/VGH cell aggregates revealed a cell arrangement typical of hepatocellular carcinoma (Fig. 2).

Plasma protein secretions from aggregated HA22T/VGH cells were analyzed and compared with those from the HA22T/VGH monolayer (Table 2). As detected by the CF method, the aggregated HA22T/VGH cells were able to synthesize and secrete all of the plasma proteins investigated, even though the density of aggregated cells was less than that of monolayer cells.

Comparison of plasma protein secretions of monolayer cells and aggregated cells of a cloned HA22T/VGH. Secretions of plasma proteins of the C5 clone of HA22T/VGH were detected in the supernatant fluid of cell monolayers and cell aggregates. Table 2 indicates that the plasma proteins secreted by C5 monolayer cells are similar to those secreted by HA22T/VGH monolayers, except that a low amount of albumin was detectable in the medium of the clone. Similarly, aggregation also induced C5 cells to secrete nearly all of the plasma proteins (Table 2).

DISCUSSION

Although the incidence of and mortality from hepatoma are very high in many countries, reports of well-established and well-characterized human hepatoma cell lines are limited, e.g., PLC/PRF/5 (2), Hep 3B and Hep G2 (1), and DELSH-5 (5). All of the biological properties of HA22T/VGH, e.g., abnormal chromosomes, high colony-forming efficiency on agar, and tumor induction in nude mice, clearly indicate the malignant nature of HA22T/VGH cells. The histological studies of nude mice tumors and cell aggregates, secretion of some induced plasma proteins, and the presence of hepatocyte-associated enzymes further indicate that HA22T/VGH originated from hepatocytes.

The phenotypic expression of hepatocyte

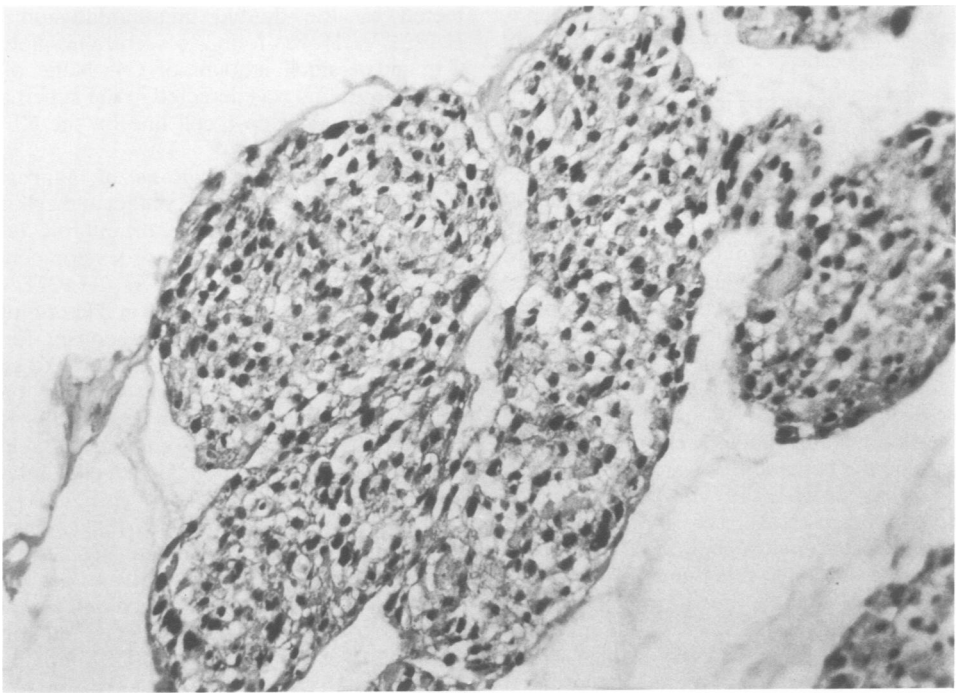

FIG. 2. Histopathological studies of aggregated HA22T/VGH cells. HA22T/VGH cells were grown as aggregates in petri dishes. The aggregated cells were fixed in Formalin and embedded in paraffin. They were then stained with hematoxylin-eosin and examined under a microscope. Magnification, ×160.

TABLE 2. Detection of human plasma protein secretion in cultured media of human hepatoma cell lines by complement fixation test

Plasma protein	Culture medium titer[a]					
	Hep G2 monolayer[b]	SK-Hep-1 monolayer[b]	HA22T/VGH monolayer[b]	HA22T/VGH aggregate[c]	C5 monolayer[b]	C5 aggregate[c]
Albumin	8,192	0	0	32	2	8
α_2-Macroglobulin	256	0	0	16	0	2
α_1-Antitrypsin	1,024	0	0	16	0	4
Transferrin	2,048	0	0	8	0	4
Haptoglobin	512	0	0	16	0	128
Ceruloplasmin	256	0	4	64	2	8
Plasminogen	16	0	0	32	0	8
C3	256	0	16	32	9	64
C4	128	0	4	16	2	256
C3 activator	8	0	16	64	36	256
α_2-HS-glycoprotein	512	0	0	16	0	8
β-Lipoprotein	64	0	0	16	0	4
α_1-Antichymotrypsin	32	0	0	16	0	4
G_c-globulin	32	0	4	64	2	128
Retinol-binding protein	128	0	0	8	0	4

[a] The 10-fold-concentrated culture fluids (25 μl) were tested for the presence of human plasma proteins by the standard CF method (4). The maximum dilutions at which a positive reaction was still observed were recorded. Numbers in each column indicate the maximum dilutions standardized to 10^6 cells at day 10.

[b] T_{75} flasks (Falcon Plastics) were seeded with 2×10^6 cells and cultured with 10 ml of DMEM containing 10% FBS, 10 mM glutamine, 100 μg of streptomycin per ml, and 100 IU of penicillin per ml. At day 10, the cells were trypsinized and counted, and the supernatant fluid was collected, centrifuged, and concentrated 10-fold by use of an ultrafiltration apparatus with a PM 10 membrane (Amicon Corp., Lexington, Mass.).

[c] HA22T/VGH cells or clone C5 cells (2×10^6 cells per 10 ml of medium) were aggregated as described in the text. The supernatants were concentrated and prepared as described in footnote *b*.

markers of HA22T/VGH was compared with other cell lines derived from human hepatoma tissues, Hep G2, Hep 3B, and SK-Hep-1. Different expressions of plasma proteins were observed in these cell lines. These results suggest that these cell lines may be arrested at different stages of cell differentiation. Hep 3B and Hep G2 are the most differentiated cells since they can secrete almost all of the plasma proteins tested. The PLC/PRF/5 cell line, which secretes seven plasma proteins as reported by Knowles et al. (12), is in the middle of the differentiation scheme. Under the same condition, HA22T/VGH secretes only five plasma proteins, perhaps indicating that it is a less differentiated hepatoma cell line. The SK-Hep-1 cell line does not secrete any plasma proteins and has spindle-shaped fibroblast-like morphology. Further studies are required to understand whether SK-Hep-1 is a dedifferentiated hepatoma cell line or not originated from liver parenchymal cells. Nevertheless, the SK-Hep-1 cells serve as a good control in the present studies.

It has been shown that cell aggregation is able to induce cell differentiation during embryonic development and in rat hepatocytes in vitro (6, 13). The expression of plasma proteins in HA22T/VGH cells likewise appears to be induced by growing these cells in aggregated form. The results indicate that HA22T/VGH cells secrete 10 additional plasma proteins in the culture medium when grown as aggregates. The changes in the secretion of plasma proteins can be due to the different regulatory mechanisms or the selective growth of cells with different phenotypes in different growth conditions. To distinguish between these two possibilities, we cloned the HA22T/VGH cells. The plasma proteins secreted by the HA22T/VGH clone C5 are similar to those found in the medium of the parent HA22T/VGH cells. As in the parental population, additional plasma proteins are secreted by C5 after cell aggregation. The secretion of α-fetoprotein may represent another differentiation marker of human hepatoma cells (1, 2, 12). Recent studies indicate that the control of synthesis of hepatitis B surface antigen and α-fetoprotein, but not albumin, is related to the growth state of Hep 3B cells (1). In contrast to other plasma proteins, α-fetoprotein is undetectable in the medium of aggregated HA22T/VGH cells. This finding further supports that α-fetoprotein biosynthesis may involve a different control mechanism. More importantly, our data strongly suggest that environmental manipulation of HA22T/VGH cells will lead to differential gene expression. Further studies to understand whether cell aggregation can induce permanent phenotypical changes are currently under way.

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