Proc. Nat. Acad. Sci. USA
Vol. 69, No. 4, pp. 956–959, April 1972

Neoplastic Transformation *In Vitro* of a Clone of Adult Liver Epithelial Cells into Differentiated Hepatoma-Like Cells Under Conditions of Nutritional Stress

(contact inhibition/agglutination/intercellular communication/karyotype/cytodifferentiation)

CARMIA BOREK

Radiological Research Laboratories, Department of Radiology, Columbia University, New York, N.Y. 10032

Communicated by Theodore T. Puck, February 4, 1972

ABSTRACT **Differentiated epithelial cells in contact-inhibited monolayers derived from adult rat liver have been transformed *in vitro* into epithelioid neoplastic cells under conditions of nutritional stress. The transformed cells maintain their differentiated quality and manufacture serum proteins. They differ from control cultures in the following properties: They are aneuploid, can be agglutinated by wheat-germ agglutinin and concanavalin A, can grow in suspension, and are able to form colonies in semisoft agar. There is no intercellular communication at permeable junctions between the cells; this is demonstrable by electrical measurement or by injection of fluorescein. The cells show invasiveness in culture, and are not inhibited by contact with normal cells. The characteristics of the hepatocytes after transformation *in vitro* resemble those of epithelioid cells derived from a transplantable hepatoma.**

Transformation of mammalian cells *in vitro* to forms that have lost contact inhibition and have acquired other properties associated with malignancy has been described (1–7). Such transformation has required action by carcinogenic agents like x-rays, neoplastic viruses, and chemical carcinogens (1–7). Moreover, the transformation has only been described for fibroblasts or fibroblast-like cells*. In the present paper, transformation by nutritional stress alone is described for a culture of differentiated rat epithelial cells.

MATERIALS AND METHODS

A cloned line of differentiated epithelial-like cells that are cuboidal was established from normal liver of a 3-month-old buffalo rat (8). The fresh tissue was minced and dissociated progressively at 37° with 0.1% trypsin, 0.1% collagenase, and 1% chick serum in Hank's salt solution that is free of Ca and Mg (Grand Island Biological Labs). After dissociation and removal of the trypsin solution by centrifugation, the cells were resuspended in F12 medium, supplemented with double the concentration of amino acids, 5% fetal-calf serum, and 1% antibiotic–antimycotic solution. 10^5 cells were plated per 100-mm petri dish (Falcon Plastics) with 10 ml of medium and were incubated in a humidified incubator at 36.5° and 5% CO_2 in air. The medium was changed three times a week. Within 20 days, the cells grew into clones. In order to obtain a homogeneous cell population of epithelial cells, free from spindle-shaped fibroblast-like cells, three consecutive clones were isolated (9); the supplemented F12 medium was used. After the third isolation, the clone of hepatocytes was grown into mass cultures (Fig. 1). Large populations were frozen in liquid nitrogen to make available an adequate supply of cells at the early stages of culture. The cells of the cloned hepatocyte line have been karyotyped as diploid†, and grew in contact-inhibited monolayers (10). Their generation time is 28 hr (9). Cells of the cloned hepatocyte line are differentiated, as indicated by their synthesis and secretion of serum proteins. The serum proteins were determined by radioimmunoelectrophoresis (11). Confluent cultures were incubated with [^{14}C]leucine (5 μCi/ml; New England Nuclear Corp.) in medium without serum for 24 hr. The medium was removed and after dialysis and lyophilization, electrophoresis was performed for 1.5 hr at 6 V/cm in veronal buffer (pH 7). Anti-whole rat serum was added and allowed to diffuse overnight. The slide was washed, dried, exposed to Kodak Royal Pan, and developed in Kodak D 19. It showed precipitin arcs of serum components that have reacted with the anti-rat antibody.

When confluent cells derived from rat kidney, lung, and heart were incubated for 24 hr in medium without serum, no serum proteins were found in the medium, as determined by acrylamide gel electrophoresis (12). Medium removed from confluent hepatocyte cultures did show bands that were stained with Coommassie blue and corresponded to bands present in the gels containing serum. Electrophoresis was performed in 7.5% gels, at 0.5 A/gel for 4 hr in Tris·HCl buffer (pH 8.9).

By the same methods used for culturing hepatocytes, a cloned hepatoma cell line was developed (8) from the Morris heptoma no. H5123 that is transplantable in buffalo rats (13). The hepatoma cells are aneuploid with an 18-hr generation time; they are not inhibited by contact with one another, and do not manufacture serum proteins (Fig. 2).

All cells were harvested from subculture with 0.1% trypsin and were grown in Dulbecco's modified Eagle's medium, supplemented with 5% fetal-calf serum and 1% antibiotic–antimycotic solution. 5×10^5 Cells were seeded in 60-mm petri dishes (Falcon Plastics) in 4 ml of medium and were incubated at 36.5° and 5% CO_2 in air. The medium was changed three times a week, unless stated otherwise.

The liver epithelial cells used in the transformation experiments had been continuously subcultured for 18 months after

* See ref. 7 for a possible exception where cells were derived from an organ culture.

† Karyotype determination with quinacrine mustard will be published elsewhere.

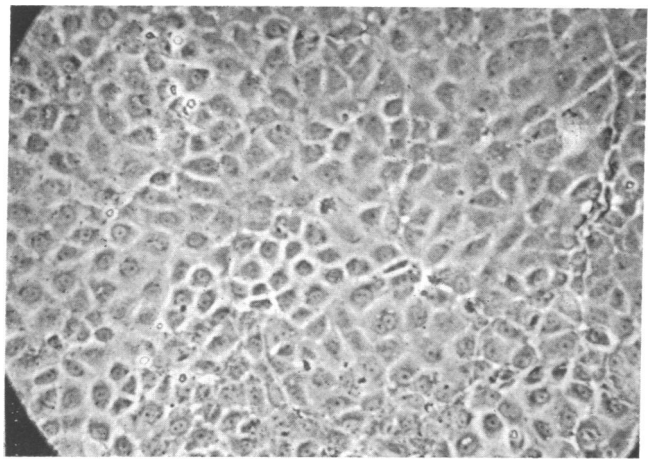

FIG. 1. Confluent monolayer of the cloned hepatocyte cell line. Phase contrast × 125.

the establishment of the cloned line. The cells were always trypsinized and harvested when they reached a density of about 10^6 in the 60-mm petri dishes so that they were never subject to crowded conditions. Experimental and control cells were seeded in the usual manner (8) into 60-mm petri dishes. Whenever the density of cells in control dishes reached 10^6 per plate, the medium was replaced by 8 ml of fresh medium. In the experimental plates, however, after the initial addition of the 8 ml of fresh medium, the medium was changed at 21-day intervals. Cell surfaces were agglutinated by concanavalin A and wheat-germ agglutinin (15).

RESULTS

After 10 consecutive days in culture without feeding, the cells appeared as a tightly packed monolayer in which no more mitosis was observed. However, within 30–40 days after the beginning of the experiment, isolated loci of refractile cells that went into mitosis and began to form a second layer in a restricted region appeared in 45 of the 50 experimental plates. No such loci were observed in the controls. The cells that developed on top of the flat, crowded epithelial monolayer remained epithelioid, and upon replication gave rise to

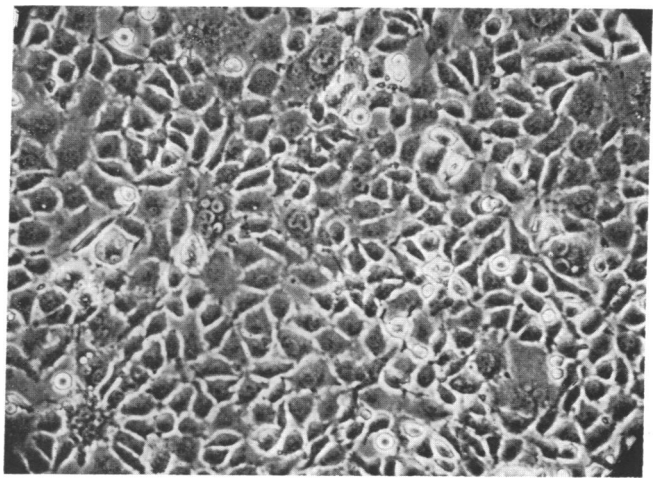

FIG. 3. Rat hepatoma cell line cultured and cloned from Morris hepatoma H5123. The cells have an epithelioid morphology, are not contact-inhibited, have a random pattern of growth, and can form multilayers in culture. Phase contrast × 125.

progeny cells that lacked the neat cuboidal structure of the liver epithelial cells, and appeared larger and irregularly oriented (Fig. 3). After 2–3 days, the refractile cells replicated rapidly, extending over the monolayer to wider areas of the plate. 5–7 Days after the initial appearance of the morphologically transformed cells, the whole petri dish was covered by a multilayer of cells (Fig. 4). This experiment was performed three times with similar results.

The medium removed from the experimental plates failed to support growth of hepatocytes that were seeded in it at densities of 5×10^5 cells, with or without adjustment of pH to 7.2, indicating that the medium in the plates, where transformation occurred, was depleted.

The karyotype of the transformed epithelioid cells is aneuploid. The generation time of the cells is 16 hr.

Serum-protein synthesis

After transformation, there was no loss in the ability of the cells to manufacture serum proteins, when compared to the

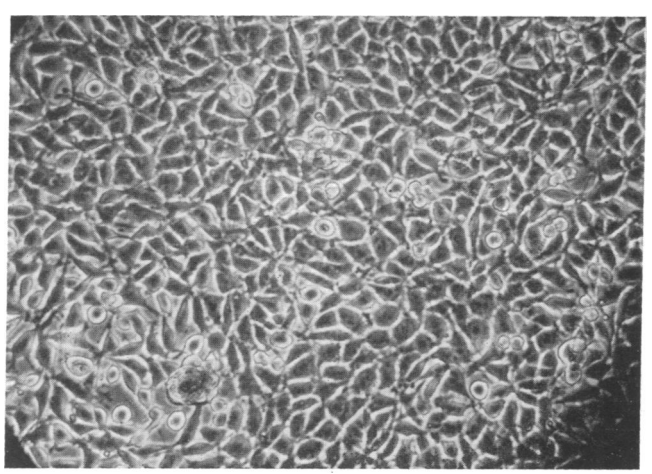

FIG. 2. Rat hepatocytes transformed *in vitro* into neoplastic cells with epithelioid morphology. The cells are not contact-inhibited, have a random pattern of growth, and can form multilayers in culture. Phase contrast × 125.

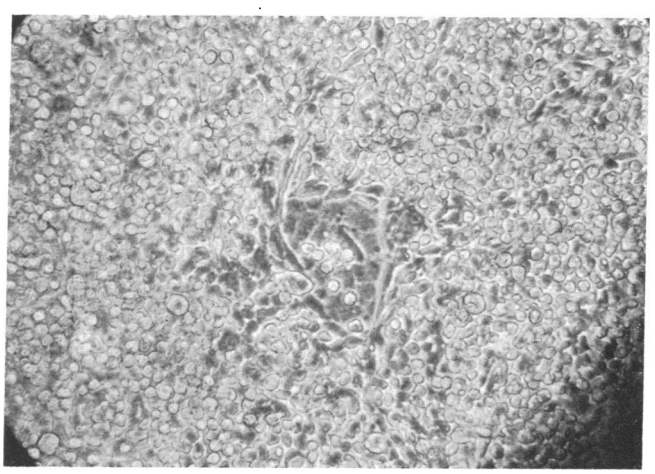

FIG. 4. The transformed hepatocytes forming multilayers over the parental normal cells. In the middle, a portion of the bottom layer can be seen with single transformed cells moving over to cover that area. Phase contrast × 125.

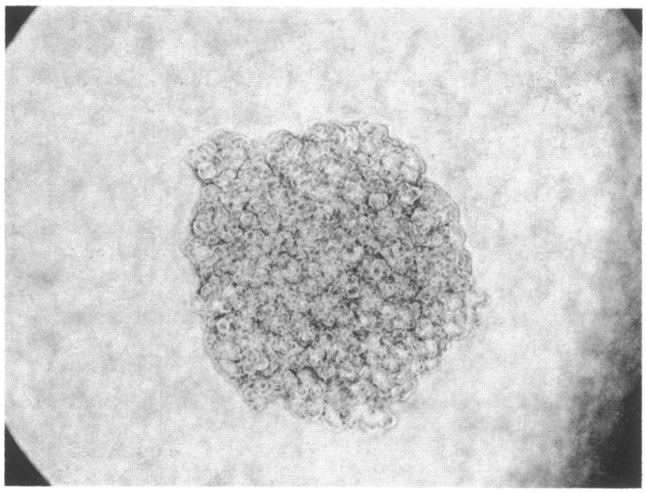

FIG. 5. An 8-day-old clone of tranformed rat hepatocytes in 0.33% agar. Phase contrast × 125.

control nontransformed liver cells, as determined by radioimmunoelectrophoresis.

Morphology, adhesiveness, and growth in suspension

The formation of multilayers of rounded cells, characteristic of these transformed epithelial cells, is distinctly different from the criss-cross pattern of transformed fibroblasts in culture (1). Another difference was the decrease in adhesiveness of the cells to surfaces on which they grew, and to one another. This could be seen both by the great facility with which trypsin detached them, in comparison with the normal liver cells, and also in their ability to grow as suspension cultures when seeded in petri dishes, in a manner similar to that of the hepatoma cell line, but in contrast to the normal cells that adhere to the dish and grow as monolayers.

Growth in agar

One of the assays for neoplastic transformation of fibroblasts has been their ability to grow in semisoft agar (14). Both the transformed liver cells and hepatoma cells display a similar ability to grow in semisoft agar. When seeded at a density of 10^2 cells per plate in 0.33% agar, made up with F12 medium and 10% fetal-calf serum, macroscopic colonies appeared within 8 days (Fig. 5). The control cells did not grow in agar.

Agglutination by plant glycoproteins

Several plant glycoproteins have been found specifically to agglutinate fibroblasts that underwent neoplastic transformation, and that were not contact-inhibited (15–18). Concanavalin A or wheat-germ agglutinin, in concentrations of 50 μg/ml, agglutinated both the transformed and the hepatoma cells but not the normal epithelial cells (Borek, C., M. Grob, & M. M. Burger, manuscript in preparation).

Intercellular communication and contact inhibition

The loss of inhibition of replication due to contact with other cells and multilayering of tumor epithelioid cells *in vitro* has previously been observed with the hepatoma cells, and was correlated with a lack of communication between the cells at their intercellular junctions, as measured by ion-current flow (8). This was in contrast to the monolayers of normal, contact-inhibited hepatocytes that did demonstrate a high permeability to the ions. It was of interest to see whether the transformed epithelioid cells described here had also lost the ability to communicate with one another. The cells after transformations were found to have no intercellular communication within the limits that can be measured by two ways: (*a*) by pulsing currents between one cell's interior and the exterior and recording the resulting voltages inside a contiguous cell (8), and (b) by injection of sodium fluorescein (19) into one cell and the observation under dark field that the dye remains localized in that cell, in contrast to nontransformed hepatocytes, where the fluorescein spreads into neighboring cells.

Contact inhibition between normal and transformed epithelial cells

Since previous findings with fibroblasts transformed *in vitro* show that their replication can be inhibited when they are cocultivated with normal cells and the reverse (20), it was striking to observe that the normal epithelial cells did not contact-inhibit the replication of the transformed cells that arose from them. A similar observation has been made with the hepatoma cells (8). Two epithelial cell lines (clones 9 and 12 derived from Wistar rat liver, kindly provided by Dr. E. A. Kaighn) were cocultivated with the transformed epithelioid cells (*a*) by seeding 0.5×10^4 transformed cells on 7-day monolayers of the normal cells that were not dividing, and (*b*) by seeding 10^5 cells of both normal and transformed cells in different areas of a petri dish that is partitioned by a glass slide and by allowing the cells to grow toward one another after removal of the barrier. The experiments were repeated three times with each cell line. In all cases, the transformed cells covered up the normal cells.

The viability of normal hepatocytes that were cultivated with transformed cells was established by cloning the cultures. Six replicate experiments were performed; the mean plating efficiency of viable cells for control cultures was 23.7% (SD ± 2.05), while the corresponding amount for the normal colonies in culture with the transformed cells was 26.5% (SD ± 2.06). Therefore, the transformed cells do not kill and replace the normal cells, but rather grow over them without affecting their viability.

Whether normal epithelial cells could replicate over a layer of transformed cells was also determined. Confluent hepatocyte cultures were labeled by incubation at 37° with 2 μCi of tritiated thymidine/4 ml of medium for 3 days, trypsinized, washed, and seeded with aliquots of 10^5 cells on (*a*) monolayers of the transformed cells, (*b*) monolayers of normal hepatocytes, and (*c*) empty plates. On each of 5 consecutive days, three plates of each combination were removed, washed, fixed in methanol, and used for radioautographic preparations. From the autoradiography it was evident that the normal hepatocytes did not replicate over the transformed cells or over monolayers of hepatocytes.

DISCUSSION

The present paper demonstrates that normal cells may be transformed to a form that displays the *in vitro* properties associated with malignancy by a simple device that involves no treatment with new agents. Transformation occurred when epithelial liver cells that were cultured for several months at low densities under optimal conditions were incubated without change of medium after having achieved confluent growth, so that the cells were exposed to the effects

TABLE 1. *Properties of cultured epithelial cells from rat liver (hepatocytes) after transformation in vitro, compared with a nontransformed culture and with a cell line cultured from rat hepatoma H5123*

	Normal liver	Transformed cells	Hepatoma cells
Morphology	Epithelial	Epithelioid	Epithelioid
Cell generation time	28 hr	16 hr	18 hr
Karyotype	Diploid	Aneuploid	Aneuploid
Ability to manufacture serum protein	Yes	Yes	No
Contact inhibition of replication	Yes	No	No
Pattern of growth in culture	Monolayers	Multilayers	Multilayers
Ability to grow in suspension cultures	No	Yes	Yes
Colony formation in 0.33% agar	No	Yes	Yes
Agglutinability of the cells by concanavalan A and wheat-germ agglutinin	No	Yes	Yes
Communication at permeable intercellular junctions	Yes	No	No
Ability to replicate over normal cells	No	Yes	Yes

of depleted medium, products of their own metabolism, lowered pH, and high proximity for a period of 6 weeks. The transformed cells remained epithelioid and differentiated. Their properties are summarized in Table 1. Although the molecular mechanisms of malignant cell transformation under different growth conditions *in vivo* and *in vitro* are still unknown (18), events that are known to occur in cells exposed to an exhausted medium and low pH (21, 22) could be postulated to trigger the conversion of normal cells into fast-dividing, aneuploid cells that have acquired new surface-membrane properties, characteristic of neoplastic cells. In particular, the occurrence of unequal division in mitosis that leads to formation of cells other than diploid have been reported in cells after a change of the nutritional environment (21); this could be the process underlying the phenomenon observed here.

The demonstration of cell transformation by means of nutritional stress would appear to have obvious possible implications for some kinds of malignancies that develop *in vivo*.

I thank Prof. J. W. Uhr, of the New York University Medical School, for some of the research facilities, to Prof. W. R. Loewenstein and Dr. R. Azarnia for their help with the cell communication studies, and to Dr. E. A. Kaighn for his help with the radioimmunoelectrophoresis. This investigation was supported by U.S. Public Health Service Research Grant CA-12536 from the National Cancer Institute.

1. Borek, C. & Sachs, L. (1966) *Nature* **210**, 276–278.
2. Vogt, M. & Dulbecco, R. (1960) *Proc. Nat. Acad. Sci. USA* **46**, 365–370.
3. Sachs, L. & Medina, D. (1961) *Nature* **189**, 457–458.
4. Stoker, M. G. P. & MacPherson, I. (1961) *Virology* **14**, 359–370.
5. Todaro, G. J., Green, H. & Goldberg, B. D. (1964) *Proc. Nat. Acad. Sci. USA* **51**, 66–73.
6. Berwald, Y. & Sachs, L. (1963) *Nature* **200**, 1182–1184.
7. Heidelberger, C. & Iype, P. T. (1967) *Science* **155**, 214–217.
8. Borek, C., Higashino, S. & Loewenstein, W. R. (1969) *J. Membrane Biol.* **1**, 274–293.
9. Ham, R. G. & Puck, T. T. (1962) *Methods Enzymol.* **5**, 90–119.
10. Abercombie, M. (1967) *Nat. Cancer Inst. Monogr.* **26**, 249–273.
11. Asofsky, R. & Thorbecke, G. J. (1961) *J. Exp. Med.* **114**, 459–470.
12. Davis, B. J. (1969) *Ann. N.Y. Acad. Sci.* **21**, 404–427.
13. Morris, H. P., Sydransky, H., Wagner, B. P. & Dyer, H. H. (1960) *Cancer Res.* **20**, 1252–1254.
14. Sanders, F. K. & Burford, B. O. (1964) *Nature* **20**, 786–789.
15. Burger, M. M. (1967) *Proc. Nat. Acad. Sci. USA* **57**, 359–366.
16. Inbar, M. & Sachs, L. (1969) *Proc. Nat. Acad. Sci. USA* **63**, 1418–1425.
17. Hsie, A. W., Jones, C. & Puck, T. T. (1971) *Proc. Nat. Acad. Sci. USA* **66**, 1648–1652.
18. Hsie, A. W. & Puck, T. T. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 358–360.
19. Loewenstein, W. R. & Kanno, Y. (1964) *J. Cell Biol.* **22**, 565–586.
20. Borek, C. & Sachs, L. (1966) *Proc. Nat. Acad. Sci. USA* **56**, 1705–1711.
21. Richards, B. M., Walker, P. M. B. & Deeley, E. M. (1956) *Ann. N.Y. Acad. Sci.* **63**, 831–848.
22. Sylven, B. (1968) *Eur. J. Cancer* **4**, 559–562.