ULTRASTRUCTURE AND CYTOCHEMICAL STUDY OF CULTURED RAT LIVER CELLS

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Epithelial cells from the weanling rat liver could proliferate easily if repeated colonial selection of the epithelial cells was performed in order to prevent overgrowth of the non-epithelial cells. The newly started two epithelial cell lines were studied ultrastructurally and cytochemically in order to examine whether or not these cells *in vitro* were hepatocytes. These cell lines possessed features common to other rat liver cell lines described in various studies. However, no definite evidence suggesting a hepatocytic origin was found, although some features were characteristic of the hepatocytes, especially those of the fetal rat. Cytochemically, these cells exhibited many types of enzyme activity, except for glucose 6-phosphatase. Cytochemical study of the cells in the primary culture suggested that the cell lines were not derived from the fully differentiated hepatocytes.

The method for establishing normal liver cell lines has recently been developed (5, 13, 15, 17, 25, 28), with many cell lines now possible. However, none of these exhibit all the morphological and functional characteristics of hepatocytes (6, 7, 12, 26), and the degree of differentiation varies among the cell lines (21). Many investigators believe that these cell lines are derived from the well differentiated hepatocytes, which show dedifferentiation as a result of unsatisfactory cultural conditions. On the contrary, Grisham *et al.* proposed that the undifferentiated hepatocytic stem cells were responsible for the origin of the cell line (8).

In the present study, rat liver cell lines which were almost identical to those described in previous studies were cultivated, with some features resembling those of hepatocytes found. Furthermore, it was found that the cells *in vitro* changed their phenotypes, which may explain the conflicting results of cell lines reported to date. However, the large discrepancy between the epithelial cells *in vitro* and the hepatocytes *in vivo* encouraged observation of the cells in the primary culture. The results supported those of Grisham *et al.* (8), namely, the successively cultivated epithelial cells may be, from the beginning, the undifferentiated precursor cells of the hepatocytes.

MATERIALS AND METHODS

Primary Culture. Male Donryu rats of 7–10 days old were sacrificed by

decapitation. The liver was extracted aseptically on a dry dish and was cut into the smallest possible pieces with apposed scalpels. After washing twice with Ca^{++} and Mg^{++} - free Tyrode solution, the settled fragments were suspended in a 0.25% solution of trypsin (Difco 1 : 250). Digestion for 30 min at 37°C was repeated three times. Digested cells were pooled and resuspended in Ham's F12 medium (Nissui) supplemented with 10% fetal calf serum (Gibco). When checked with trypan blue, the viable cells numbered more than 90%. Fifty thousand cells were inoculated in a 6 cm petri dish containing 3 ml of medium, and incubated under 5% CO_2 humidified air at 37°C. The unattached cells were transferred to new dishes after one hour of incubation in order to remove the nonepithelial cells which had quickly attached to the glass surface (25).

Colonial Selection. After decantation of the medium, pieces of filter paper which had previously been immersed in a trypsin solution were placed on the growing foci of the epithelial cells and incubated for five min. The filter papers, to which the digested cells attached, were transferred into the new dishes containing the prewarmed complete medium. Epithelial cells formed many colonies in the new dishes. This procedure was repeated to eliminate the non-epithelial cells. The resultant two cell lines derived from two separate rats were named RL1 and RL2, and were investigated in the present study. A simplified replicate culture was prepared in order to examine the population doubling time (16). At the time of the present experimentation, the approximate ages of the two cell lines, RL1 and RL2, were 7 months and 3 months respectively.

Ultrastructure and Cytochemistry. For electron microscopy, the cells were fixed, embedded and sectioned with conventional procedures either *in situ* on cover slips or as pellets after trypsinization. Ultrathin sections were stained with uranyl acetate followed by lead citrate.

The cells cultured on cover slips were rapidly dried in a cooled room and investigated cytochemically without fixation, except for the examination of PAS reaction and Sudan III. The enzymes examined were as follows; glucose 6-phosphatase (24), adenosine triphosphatase (24), 5'-nucleotidase (24), alkaline phosphatase (4), acid phosphatase (2), β -glucuronidase (9), phosphorylase (22), non-specific esterase (11), succinate dehydrogenase (18), lactate dehydrogenase (18), and glucose 6-phosphate dehydrogenase (20). Cells in the primary culture were studied at the 3rd, 5th and 7th day *in vitro*. Because many types of cells were present in the primary culture, each colony formed in the primary culture was photographed using phase contrasted microscopy, then examined cytochemically to compare the light microscopic appearance with cytochemical properties.

RESULTS

Two types of epithelial cells were observed in the primary culture, namely, granular and agranular cells. Only the agranular cells expanded broadly. The resultant two cell lines appeared to consist of this type of cell. The present two cell lines showed almost the same light microscopic findings. Morphology under phase contrasted microscopy was that of "epithelial" cells (Fig. 1), that is, the polygonal cells with a clear halo forming pavement patterns. The periphery of the colony was smooth. The cells had a relatively broad cytoplasm with a few granules and

FIG. 1. Phase contrasted microscopy shows the confluent epithelial cells at the 10th passage of RL1, which are polygonal in shape and form the pavement pattern. $\times 510$

oval nuclei. Cells with two nuclei were approximately 7% under the confluent state, but they were variable and fewer in number under the preconfluent state. Cells with three or more nuclei were never found. The average number of nucleolus were 2.3/nucleus. A cellular stream was observed which resembled the hepatic cord at times. After confluency they remained in monolayer and continued to proliferate. Population doubling time of these cell lines was 24–30 hours.

As seen by electron-microscopy, the proliferative cells were arranged side by side, with well-developed tight junctions and interdigitations, reflecting the epithelial nature (Fig. 2). Stud-like junctions were also found (Fig. 3). There were numerous microvilli at the cytoplasmic membrane of the detached cells by trypsin. The microvilli between the two tight junctions were similar to those of bile canaliculi. However, since no integrated structures were noted, these structures were not con-

FIG. 2. Electron micrograph of RL1 at the 10th passage. The cells are cut in situ parallel to the glass surface. Note the well developed tight junctions (arrows). $\times 7,700$

FIG. 3. Electron micrograph of RL1 at 40th passage. The cells were pelleted after short trypsinization. Stud-like junctions (arrows) as well as tight junctions remain, in spite of partial dissociation of the cell contact. $\times 13,800$

TABLE 1. *Cytochemical enzyme activities of two rat liver cell lines*

Enzymes	RL1	RL2
Phosphorylase	++	++
Alkaline phosphatase	—*	++
Acid phosphatase	++	++
β -glucuronidase	+	++
Adenosine triphosphatase	++	++
5'-Nucleotidase	+	++
Glucose 6-phosphatase	—	—
Esterase (Indoxyl)	—	+
Succinate dehydrogenase	++	++
Lactate dehydrogenase	++	++
Glucose 6-phosphate dehydrogenase	++	++

* Alkaline phosphatase of RL1 was positive until 19 passages.

firmed to be bile canaliculi. Nuclei were round with uniformly dispersed chromatin and prominent nucleoli. The cytoplasm was very similar to those of the immature fetal hepatocytes. Immaturity was indicated by the free ribosomes, the low degree of development of rough endoplasmic reticulum, and the absence of smooth endoplasmic reticulum and glycogen.

FIG. 4. Cytochemistry of RL1 (a–e) and hepatocytes in primary culture (f). Phosphorylase (a), lactate dehydrogenase (b), alkaline phosphatase (c), 5'-nucleotidase (d), adenosine triphosphatase (e), and glucose 6-phosphatase (f).

Rough endoplasmic reticulum was represented by considerably dilated cisterns containing electron opaque materials. The ribosomes were numerous and many ribosomes were clustered. Many Golgi apparatuses were scattered and were highly developed (Fig. 3). Lysosomes, including multivesicular bodies, and lipid droplets, were relatively abundant. Mitochondria were numerous and had cristae which traversed the entire width of the organelle. Microbodies were not seen.

The bundles of 100 Å fibrils were sometimes scattered throughout the cytoplasm. These fibrils appeared those which are usually present in the cytoplasm of *in vitro* cells, maintaining the cellular structure. On the other hand, a concentration of cytoplasmic 80 Å fibrils was seen adjacent to the plasma membrane. Furthermore, thick fibrils were accumulated at the extracellular space. Periodicity was not found in these fibrils.

Cytochemical studies revealed that the present two cell lines retained many types of enzymatic activity. These are summarized in Table 1. Phosphorylase activity was found throughout the cultivation (Fig. 4a), although glycogen was not demonstrated. Activities of lactate dehydrogenase (Fig. 4b), succinate dehydrogenase and glucose 6-phosphate dehydrogenase were high. The activity of alkaline phosphatase was localized within the total plasma membrane (Fig. 4c). It is interesting to note that the activity of alkaline phosphatase disappeared at the 40th passage in RL1, although it was demonstrated at the 10th passage. At the 19th passage, the alkaline phosphatase negative cells were intermingled among the positive cells. The activity of 5'-nucleotidase was found on the plasma membrane (Fig. 4d) and, in some colonies, within the cytoplasm. Cells without this enzyme were found among the positively stained cells. Adenosine triphosphatase activity was localized mainly at the mitochondria (Fig. 4e). Acid phosphatase activity was high. Generally, the confluent area had high enzymatic activities. Mitotic cells also appeared to have high activities. Obviously, these findings cannot consistently indicate the true activities of each cell because of varying cellular thickness. The activity of glucose 6-phosphatase was not demonstrated.

In the primary culture, glucose 6-phosphatase was found only in the granular type of epithelial cells (Fig. 4f). Because glucose 6-phosphatase is a specific enzyme for hepatocytes in the liver, the granular cells were thought to be hepatocytes. These cells, however, did not appear to proliferate, because colonies formed by this type of cell did not expand their area. Mitotic figures were also very rare. Furthermore, these cells showed degenerating features. Many vacuoles were seen in the cytoplasm, and tremendous lipid droplets were demonstrated cytochemically in these cells. On the contrary, the epitheloid cells with few granules proliferated rapidly, and many mitotic figures were noted in these colonies. These colonies, from the beginning, did not have glucose 6-phosphatase activity.

All types of cells in the primary culture showed phosphorylase activity, although this enzyme was exclusively high in hepatocytes *in vivo*. Lactate dehydrogenase and glucose 6-phosphate dehydrogenase activity were high only in granular cells, with other types of cells showing weak activity. Alkaline phosphatase activity was positively demonstrated in granular cells and endothelial-like cells. The agranular type of epithelial cells and spindle cells had no alkaline phosphatase activity.

DISCUSSION

Initially, one needs to confirm distinctions of the present study's cell lines and cell lines discovered in other studies, in reference to the origin of the epithelial cells *in vitro*. At present, however, no clear statement exists regarding the origin of the cell line, since the immaturity of the cell lines may be the only common feature. Reports to date have revealed some evidence in that the cell lines may be derived from hepatocytes. Diamond *et al.* reported tyrosine aminotransferase and glucose 6-phosphatase activity in their cell lines although the activities were at very low levels (6). The transformed cells, following an exposure to chemical carcinogens, produced tumors in animals, some of which were similar to hepatocellular carcinomas (27). The present cell lines had ultrastructural features similar to those of the fetal rat hepatocytes (3). Discrepancy between the cells *in vitro* and hepatocytes *in vivo* may be simply explained by the morphological and functional dedifferentiation after the adaptation to the cultural conditions. Retention of the differentiation was possible in organ culture (19). However, the organ culture allowed no proliferation of cells except for the tumor cells in organotypic cultures (29). Furthermore, there have been no reports on the redifferentiation of the rat liver cell lines in organ culture.

On the other hand, ultrastructural study of the hepatocytes in primary culture showed that even in senescent cultures, the hepatocytes showed no structural simplification (1). Hepatocytes transferred *in vitro* after partial hepatectomy could not enter the S phase (10). Single cell cloning in the primary culture was reported (5), but identification of the cell from which the clone developed was not demonstrated. The present cytochemical study showed that differentiated hepatocytes with glucose 6-phosphatase had no proliferation. The growing epithelial cells in primary culture had no glucose 6-phosphatase activity from the beginning.

The present cell lines possessed many types of enzyme activities, excluding only glucose 6-phosphatase. Williams *et al.* also found their cell lines exhibited various enzymes, and reported that cells *in vitro* were identical with hepatocytes because other cells present in the liver, including bile epithelial cells, were cytochemically negative in many enzymes (26). However, it is not possible to compare the cells *in vitro* with *in vivo* cells in the present study simply because normal cells may show quite different phenotypes when they are cultured. Phosphorylase, which is exclusively high in activity in hepatocytes cytochemically, as well as lactate dehydrogenase, were found in all types of cells in the primary culture of the present study. Alkaline phosphatase seemed specific to some cells, but the activity was unstable. In the present cell lines, the activity of adenosine triphosphatase was localized at the mitochondria. It was negative or very weak in activity at plasma membrane. Karasaki *et al.* observed that adenosine triphosphatase activity on the external surface of normal diploid cell lines was minimal, whereas a considerably high activity was found in all tumorigenic cell lines tested (14).

Morphologically, the present cell lines showed epithelial nature. Although known epithelial cells in the liver are hepatocytes and bile epithelial cells, some undifferentiated cells may be present in the liver as suggested by Grisham *et al.* (8). When short liver perfusion with collagenase was conducted, only central zones of the lobules were digested. This method yielded only hepatocytes, and when they

were cultured, no agranular cells appeared (23). Therefore, the undifferentiated cells may be situated in the periphery of the lobules as the precursor cells of hepatocytes.

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