Morphological and Functional Studies of Human Fetal Liver Cells in Primary Monolayer Culture

OSAMU NAKAGOMI and NAKAO ISHIDA

Department of Bacteriology, Tohoku University School of Medicine, Sendai 980

NAKAGOMI, O. and ISHIDA, N. Morphological and Functional Studies of Human Fetal Liver Cells in Primary Monolayer Culture. Tohoku J. exp. Med., 1980, 132 (3), 277-287 — Livers from human fetuses between the 16th and 24th weeks of gestation were dissociated by successive dispase and collagenase digestion followed by two cycles of low-speed differential centrifugation. This improved method recovered approximately $1 \times 10^8$ cells (90% hepatocytes and 90% viable cells) from 4 g of liver tissue. These hepatocytes were set into primary culture and monolayer granular hepatocytes were obtained within a week. Both albumin and α-fetoprotein production was demonstrated in these granular hepatocytes by the immunoperoxidase method for 2 weeks and α-fetoprotein production in the culture medium occurred for a week by the single radial immunodiffusion method. The morphological features of the granular hepatocytes could be distinguished from those of the other type of epithelial cells with clear cytoplasm. During the cultivation period, gradual changes from granular to clear hepatocytes with high mitotic activity were found. — human fetal liver; primary culture; α-fetoprotein; albumin; immunoperoxidase technique

Cell biologists have long attempted to obtain functionally intact liver parenchymal cells from the normal human fetal liver for primary culture (Zuckerman et al. 1967; Bissell and Tilles 1971; Noyes 1973; Watanabe et al. 1976; Ishida et al. 1978; Tsiquaye et al. 1978). For biochemical investigations it is necessary to obtain homogeneous liver parenchymal cell cultures. However, the contamination of hematopoietic cells and fibroblasts seems to be unavoidable according to previously published methods; the presence of the former produces unfavorable culture conditions in the early phase and the overgrowth of the latter hampers the long-term cultivation and serial propagation of liver parenchymal cells. The present report describes an improved and reliable method for obtaining homogeneous viable liver parenchymal cells with relatively high yields using successive dispase and collagenase digestion and cycles of low-speed differential centrifugation. The resulting hepatocyte suspension set into primary culture was studied both morphologically and functionally using two liver cell marker proteins, i.e. albumin and α-fetoprotein (AFP).

Received for publication, September 10, 1979.
This investigation was partly supported by grants from the Ministry of Health and Welfare, Hepatitis Research Committee and the Ministry of Education, Science and Culture, Japan.
MATERIALS AND METHODS

Tissue. Human fetal livers were obtained from fetuses delivered by artificial abortion for therapeutic purposes. The gestational age of the fetuses varied from 16 to 24 weeks. The aseptically removed livers were transported to our laboratory in chilled RPMI 1640 medium containing 100 U/ml of penicillin and 100 µg/ml of streptomycin and stored in a refrigerator until use. The elapsed time between removal of the liver and preparation of the culture varied between 2 and 10 hr.

Tissue culture medium. The standard growth medium consisted of RPMI 1640 (Grand Island Biological Company, Grand Island, New York, USA) buffered with sodium bicarbonate, containing 100 U/ml of penicillin, 100 µg/ml of streptomycin and supplemented with 10% fetal bovine serum (Flow Laboratories, Stanmore, N.S.W., Australia). Further supplementation with dexamethasone (from 10 to 50 µg/ml) was evaluated.

Dissociation and preparation of liver parenchymal cells. A portion of liver tissue was rinsed once with RPMI 1640 medium after the visceral peritoneum and the hilar components had been carefully removed, and was minced into about 1 mm³ fragments with sharp razors in RPMI 1640 medium containing 1,000 PU/ml of dispase™ (Godo Shusei Co., Ltd., Tokyo, Japan). The fragments were transferred to a glass centrifuge tube containing 40 ml of the enzyme solution and then incubated in a waterbath with occasional agitation for 30 min at 37°C. The digested fragments were allowed to settle by gravity for 10 min and the supernatant which consisted mainly of erythrocytes, erythroblasts, some damaged hepatocytes and tissue debris was discarded. The precipitate was suspended in a small volume of RPMI 1640 medium and then pressed gently with the plunger of a 10-ml plastic syringe through a sterile commercially available tea strainer into 20 ml of RPMI 1640 medium. After adding 20 ml of RPMI 1640 medium containing 0.1% collagenase (Sigma type ‡W, Sigma Chemical Company, St. Louis, Missouri, USA), the partially dispersed tissue fragments were transferred to a 40 ml centrifuge tube and incubated for 30 min at 37°C with gentle but more frequent agitation to prevent tissue reaggregation. The digested fragments were again allowed to settle by gravity for 10 min and the resulting sediment was dispersed with a small volume of the growth medium by several cycles of careful pipetting and suspended in 40 ml of the growth medium without serum and centrifuged for 2 min at 400 rpm at 4°C. This differential centrifugation was repeated 2 times and the resulting cell pellets were finally suspended in appropriate amounts of the growth medium to give a final cell concentration (exclusive of the erythroid series of the cells) of approximately 5 × 10⁴/ml.

Preparation of monolayer cultures. One ml portions of the final cell suspension were seeded into 35×15 mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) with and without sterile glass coverslips. The inoculated cells were allowed to adhere in a humidified atmosphere of 5% CO₂ and 95% air at 37°C for one day, after which the initial medium was aspirated and 2 ml of fresh growth medium were added. The cultures were maintained under the same conditions and a complete change of medium was performed every other day. From about 4 g of liver tissue, a batch of 20 monolayer cultures was obtained.

Confluent primary monolayer cultures of liver parenchymal cells were subcultured at various split ratios by the routine trypsin (0.05%)-EDTA (0.02%) treatment for 5 min after washing the monolayer with phosphate buffered saline (0.01 M, pH 7.2: PBS) containing EDTA (0.02%).

Morphological and immunohistochemical observation of the cultured cells. Morphological observations were made by phase-contrast microscopy every day during cultivation. Freshly prepared cell suspensions were smeared on coverslips and fixed in Carnoy’s solution or absolute methanol and stained with hematoxylin and eosin (H.E.) or Giemsa solution.

For the detection of intracellular albumin and AFP, the indirect immunoperoxidase technique was used. The cells cultured on coverslips were harvested at various stages and fixed for 10 min in absolute acetone, dried and incubated for 1 hr at 37°C with rabbit anti-human albumin serum (Behringwerke AG, Marburg, West Germany) or rabbit anti-human AFP serum (Behringwerke AG, Marburg, West Germany), then washed in PBS.
Human Fetal Liver Cells in Primary Monolayer Culture

and next incubated for 30 min at 37°C with horseradish peroxidase conjugated goat anti-rabbit IgG γ-globulin (Miles Laboratories Inc., Elkhart, Indiana, USA). The coverslips were then washed in PBS and incubated in diaminobenzidine with peroxide (Graham and Karnovsky 1966), dehydrated, and mounted. The specificity of the staining was tested concurrently using a control preparation treated with non-immune rabbit serum instead of anti-albumin and anti-AFP serum.

Quantitation of AFP and proteins. The culture medium was harvested at the time of each medium renewal and centrifuged for 10 min at 3,000 rpm. The supernatant was concentrated 50-fold by using Minicon™-B15 (Amicon Corporation, Lexington, Massachusetts, USA) and the AFP titers were assayed by the single radial immunodiffusion method (Mancini et al. 1965). Protein was measured by the microbiuret method (Itzhaki and Gill 1964) after scraping the cells from the substratum and lysing with 10 M sodium hydroxide.

RESULTS

Cell suspension obtained by the method described here contained a large number of hepatocytes, approximately 90% of which excluded trypan blue, and a small number (less than 10%) of contaminating hematopoietic cells. The hepatocytes were identified by their round, clear nuclei and large eosinophilic cytoplasm (Figs. 1 and 2). Many stages of the developing erythroblasts were seen and identified by their typical morphology of dark, chromatin rich, spherical nuclei without nucleoli and small round cytoplasm which stained basophilic, polychromic and eosinophilic with Giemsa stain.

Intermediate-sized cells having large spherical clear nuclei with one or two nucleoli and scanty round basophilic cytoplasm were sometimes observed and considered to be proerythroblasts but the possibility of their being precursor hepatocytes could not be completely neglected. A very small number of cells with dark elongated or oval shaped nuclei and various shapes of cytoplasm were considered to be fibroblasts. Kupffer and endothelial cells were thought to be included in unidentified cells.

Indirect immunoperoxidase stainings were performed to demonstrate the existence of albumin and AFP which are indicators of human fetal liver parenchymal cells. The specific staining of both albumin and AFP was demonstrated in the cytoplasm of the large size cells but no significant staining was observed in the erythroid series of cells (Fig. 3). This result indicated that the large size cells in the preparation were liver parenchymal cells that had preserved synthesized proteins in their cytoplasm during the preparation steps.

Two cycles of differential low-speed centrifugation employed at the final step of the preparation enabled us to recover liver parenchymal cells with high purity (90% hepatocytes) and good viability (90% viable cells). Table 1 shows that many erythroblasts, nonparenchymal cells and damaged hepatocytes were removed by the differential centrifugation. Liver parenchymal cells of higher purity could be obtained by additional repeated cycles of differential centrifugation if a little attention was paid to the cell yield. However, it was difficult to obtain a yield of more than 90% viable cells in the final preparation probably because new damage was inflicted on cells at the time of resuspending the sedimented cell packs.
Fig. 1. Initial cell suspension stained with H.E. Many erythroblasts (small darkly stained cells) are seen among liver parenchymal cells.

Fig. 2. Final cell suspension stained with H.E. Dispersed cells are mainly hepatocytes. Erythroblastic contamination is scarce.

Fig. 3. Final cell suspension stained with anti-AFP by the immunoperoxidase method. Note the cytoplasmic staining seen in almost all the isolated hepatocytes.

Fig. 4. Immunoperoxidase staining for albumin in 2 day culture cells. Hepatocytes show positive staining but contaminating fibroblasts are not stained.
TABLE 1. Characteristics of liver cell suspension

<table>
<thead>
<tr>
<th></th>
<th>Initial cell suspension</th>
<th>Final cell suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total nucleated cell number</td>
<td>$5 \times 10^7$</td>
<td>$1 \times 10^7$</td>
</tr>
<tr>
<td>Cell composition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parenchymal</td>
<td>36%</td>
<td>91%</td>
</tr>
<tr>
<td>Hematopoietic</td>
<td>60%</td>
<td>8%</td>
</tr>
<tr>
<td>Unclassified</td>
<td>4%</td>
<td>1%</td>
</tr>
<tr>
<td>Viability</td>
<td>75%</td>
<td>90%</td>
</tr>
</tbody>
</table>

Cell suspensions were obtained from 4 g liver tissue. Cell composition was estimated by counting 500 cells of a smear preparation. Viability is the percent of trypan blue excluded cells.

Immediately after seeding, many isolated hepatocytes and some clusters of hepatocytes consisting of less than 8 cells were observed in contact with each other. These spherical cells adhered to the substratum of the dishes and began to flatten during the initial 24 hr of incubation. The first medium renewal was performed at this time to remove unattached cells which would otherwise undergo degeneration. Spreading of the hepatocytes on the substratum was not completed until a further 24 hr had elapsed. The dexamethasone seemed to accelerate the attachment and flattening of hepatocytes. So it was added routinely to the growth medium.

The cells spread over the bottom of the dishes had granular cytoplasm of polygonal contour and round clear nuclei with one or two spherical nucleoli located in the center of the cells. Both albumin and AFP were demonstrated in the majority of the hepatocytes by the indirect immunoperoxidase technique (Fig. 4).

On the 4th day of cultivation the hepatic cell aggregates began to approach one another to form continuous monolayer cell sheets in which the polygonal-shaped hepatocytes were apposed to one another in straight line junctions (Fig. 5). The indirect immunoperoxidase technique revealed both albumin and AFP in the cytoplasm of some hepatocytes in one week old culture (Figs. 11 and 12).

In accordance with these morphological findings, AFP was detected in the medium up to the 7th–9th day of cultivation, but the quantities of AFP in the medium markedly decreased after 7 days of incubation (Table 2).

Scattered nonviable cells identified by their amorphous structure that lacked cytological detail were occasionally observed on the monolayer surface as cultures progressed beyond one week. Fibroblasts, more exactly, spindle shaped cells with dark, oval-shaped nuclei occasionally appeared at the periphery of hepatocyte aggregates around the end of the 2nd week and once they had appeared, rapidly began to proliferate during the 3rd week of culture. This fibroblastic proliferation could not be significantly suppressed by the addition of dexamethasone at concentrations from 10 to 40 µg/ml to the growth medium. However, 50 µg/ml of dexamethasone in the medium seemed to cause the deterioriation of the whole culture. A gradual decrease in the number of albumin and AFP producing cells was observed in the liver parenchymal cell monolayer cultures. Less than 1% of
TABLE 2. AFP concentration in the culture medium of human fetal liver parenchymal cell primary culture

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>AFP (ng/ml)</th>
<th>Cellular proteins (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>240</td>
<td>718</td>
</tr>
<tr>
<td>1-3</td>
<td>160</td>
<td>530</td>
</tr>
<tr>
<td>3-5</td>
<td>140</td>
<td>485</td>
</tr>
<tr>
<td>5-7</td>
<td>100</td>
<td>520</td>
</tr>
<tr>
<td>7-9</td>
<td>40</td>
<td>603</td>
</tr>
<tr>
<td>9-11</td>
<td>0</td>
<td>621</td>
</tr>
</tbody>
</table>

Liver parenchymal cells were seeded at a concentration of approximately $5 \times 10^5$/ml (1 mg cellular protein) and cultured as described in "Materials and Methods". The culture medium was concentrated 50-fold and the AFP content assayed by the single radial immunodiffusion method. Cellular protein was estimated by the micro-biuret method.

the cells could produce either of these proteins at the end of the 2nd week and no positive staining was detected in the 3rd week of culture. However, the morphology of the hepatocytes with granular cytoplasm was well preserved and the majority of the cells were viable during this period (Fig. 7).

A noticeable change in the 4 week old culture was that the dark granules in the cytoplasm of the hepatocytes gradually decreased in number and began to aggregate in the perinuclear cytoplasm (Fig. 8). This apparent cellular alteration from granular hepatocytes to clear hepatocytes was also observed when the 2nd week old cultures were subcultured at split ratios of 1:2 or more. The morphology of the granular hepatocytes was well preserved only when a scaled-down culture was performed in which cells from two dishes were transferred into one dish. The clear hepatocytes had neither detectable albumin nor AFP in their cytoplasm but their morphological appearance was completely different from that of the fibroblasts. The former had clear round nuclei with one or two nucleoli and had a cobblestone appearance (Fig. 9). The latter had dark elongated nuclei, characteristic spindle-shaped cytoplasm and were arranged in bundles (Fig. 10). The fibroblastic proliferation was also accelerated by subcultivation with split ratios of 1:2 or more. It could not be predicted which type of cells would predominate but in general fibroblastic growth occurred more frequently. It is, however, of interest that a group of epithelial-like cells which resembled the clear hepatocytes rapidly proliferated and survived for more than 400 days in culture. Although the survival time of the cells was variable among the cultures, both clear hepatocytes and fibroblasts survived for approximately 100 days, i.e. the proliferation continued up to the 8th to 10th passage.

**DISCUSSION**

Human fetal livers obtained from fetuses aged between 16 and 20 weeks of gestation comprised about 45% liver parenchymal cells and 45% hematopoietic cells, and the latter consisted almost entirely of erythroblasts (Thomas and Yoffey 1964). One of the difficulties in obtaining a homogeneous liver parenchymal cell
Fig. 5. Human fetal liver parenchymal cells set into primary culture. Phase-contrast micrograph of living cells. 4-day-old culture.

Fig. 6. 10-day-old culture, otherwise as in Fig. 5.

Fig. 7. 15-day-old culture, otherwise as in Fig. 5. Granular epithelial nature of the hepatocytes was preserved.

Fig. 8. 23-day-old culture, otherwise as in Fig. 5. Note decrease in number and aggregation to perinuclear cytoplasm of dark granules in the cytoplasm.

culture lies in the difficulty of the cell separation technique to exclude the contaminating erythroblasts without damaging the parenchymal cells. Bissell and Tilles (1971) reported that a typical preparation of trypsinized human fetal liver
contained both hematopoietic cells and hepatic parenchymal cells in a ratio of approximately 100:1. The final cell suspension obtained by the method described in this report contained 90% liver parenchymal cells. This meant that the hematopoietic cells contained could selectively be removed at the time of the first
medium renewal and obtained a homogeneous liver parenchymal cell suspension. Noyes (1973) stated that formation of cells in the granulocytic and erythrocytic series was prominent in the first 2 weeks of culture. We also experienced the spreading out of the erythroblasts and, less frequently, granulocytic cells when the initial cell suspension was inoculated. Whether or not there occurred real erythropoiesis was, however, questionable and the possibility of the release of many stages of developing erythroblasts from the explants should be explored.

Another constantly encountered problem was fibroblastic contamination in the liver cell preparation and overgrowth by the contaminant in the advanced stage of the culture. Cycles of low-speed differential centrifugation employed in this study enabled the separation of fibroblasts from hepatocytes because fibroblasts were smaller than hepatocytes in size and lower in weight. Since this exclusion of fibroblasts was not complete, they appeared and proliferated at around the end of the 2nd week of culture. Very recently Tsiquaye et al. (1978) adapted the method of differential attachment to minimize the fibroblastic contamination and obtained homogeneous hepatocyte culture. However, they also experienced the appearance of some spindle-shaped cells in the culture. Noyes (1973) reported that the addition of 20 μg/ml of hydrocortisone to the culture media resulted in a marked curtailment of fibroblastic growth. Contrarily, Tsiquaye et al. (1978) experienced fibroblastic proliferation even in the presence of hydrocortisone (200 μg/ml) and dexamethasone (10–40 μg/ml) which we also experienced. The present authors have, however, no intention of contradicting what has been observed previously regarding the effects of these steroids on the growth of fibroblasts because fibroblastic growth was significantly rapid and vigorous in the absence of these agents especially at the very early stage of culture.

The liver parenchymal cells set into the primary culture showed the typical granular epithelial nature of the hepatocytes reported by other workers (Bissell and Tilles 1971; Guillouzo et al. 1972; Noyes 1973; Tsiquaye et al. 1978). This morphological integrity of the hepatocytes was well preserved for 3 weeks in culture. The intracytoplasmic presence of albumin and AFP was demonstrated by the indirect immunoperoxidase technique up to the 2nd week in culture although the positively stained hepatocytes rapidly decreased in number during the 1st and 2nd weeks. In accordance with the immunohistochemical evidence, quantitation by the single radial immunodiffusion method revealed that AFP had been released into the culture medium during the first 7–9 days in culture. These results indicated that some of the hepatocytic functions were preserved for about 2 weeks in this in vitro culture system.

Loss of the capacity to secrete AFP into the culture medium was apparently associated with the cellular alteration from granular to clear hepatocytes. Neither albumin nor AFP was detected in the cytoplasm of the clear hepatocytes by the indirect immunoperoxidase technique. The clear hepatocytes that appeared in our culture seemed to correspond to the clear epithelial cells described by Guillouzo et al. (1972). According to their ultrastructural observations the
clear epithelial cells seemed to originate from dedifferentiated hepatocytes although some other possibilities could not be overlooked. Concerning cell strains derived from morphologically clear hepatocyte, we found one strain that survived for more than 400 days and two strains that survived for approximately 120 days. The appearance of such clear epithelial cells with high mitotic activities has also been observed in the cultures of adult rat livers (Grisham et al. 1975; Wanson et al. 1977; Miyazaki 1978). Several cell lines with epithelial morphology were established in adult rat liver cell cultures (Sato et al. 1968; Iype 1971; Williams and Gunn 1974; Williams 1976; Tsutamune 1975), and some workers considered that these cell lines might be derived from clear epithelial cells (Miyazaki 1978; Tanaka and Ichihara 1978).

Many workers have now succeeded in obtaining morphologically similar or identical primary cultures from human fetal livers. It has become necessary to characterize the morphology and function of these primary cultured hepatocytes in vitro. In such studies, the importance of obtaining the homogeneous liver parenchymal cell suspension and setting it into primary culture should be stressed. This requirement can partly be satisfied by the method described in this report as well as the method reported by Tsiquaye et al. (1978).

One of the major objectives of establishing human liver parenchymal cell cultures is to establish a readily reproducible culture system which permits the replication of hepatitis B virus (HBV). Infection experiments of HBV were repeatedly performed in the culture system described here but always resulted in an unfavorable outcome. HBV may require for its replication either some replication factors which exist in hepatocytes in vivo but not in vitro or a longer incubation period during which hepatocytes in the culture should remain functionally active.

References


