In Vitro Cell. Dev. Biol.—Animal 35:501–509, October 1999

1071-2690/99 $05.00+0.00

INDUCTION OF THREE-DIMENSIONAL ASSEMBLY OF HUMAN LIVER CELLS BY SIMULATED MICROGRAVITY

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(Received 16 October 1998; accepted 30 April 1999)

SUMMARY

The establishment of long-term cultures of functional primary human liver cells (PHLC) is formidable. Developed at NASA, the Rotary Cell Culture System (RCCS) allows the creation of the unique microgravity environment of low shear force, high-mass transfer, and 3-dimensional cell culture of dissimilar cell types. The aim of our study was to establish long-term hepatocyte cultures in simulated microgravity. PHLC were harvested from human livers by collagenase perfusion and were cultured in RCCS. PHLC aggregates were readily formed and increased up to 1 cm long. The expansion of PHLC in bioreactors was further evaluated with microcarriers and biodegradable scaffolds. While microcarriers were not conducive to formation of spheroids, PHLC cultured with biodegradable scaffolds formed aggregates up to 3 cm long. Analyses of PHLC spheroids revealed tissue-like structures composed of hepatocytes, biliary epithelial cells, and/or progenitor liver cells that were arranged as bile duct-like structures along nascent vascular sprouts. Electron microscopy revealed groups of cohesive hepatocytes surrounded by complex stromal structures and reticulin fibers, bile canaliculi with multiple microvilli, and tight cellular junctions. Albumin mRNA was expressed throughout the 60-d culture. A simulated microgravity environment is conducive to maintaining long-term cultures of functional hepatocytes. This model system will assist in developing improved protocols for autologous hepatocyte transplantation, gene therapy, and liver assist devices, and facilitate studies of liver regeneration and cell-to-cell interactions that occur in vivo.

Key words: human hepatocytes; microgravity; bioreactor; biodegradable scaffolds; cell cultures.

INTRODUCTION

Hepatocytes are unrivaled by any other parenchymal cell type in functional diversity and complexity. These cells divide only one or two times throughout the normal lifespan of a mature liver. However, hepatocytes exhibit an extraordinary regenerative ability in response to liver injury (5). It has been exceedingly difficult to establish long-term cultures of hepatocytes in vitro. Previous efforts to do so have failed when either plastic or collagen-coated rigid plates were used (17). Improved growth was achieved by culturing hepatocytes between two layers of collagen (22). However, all of these culture systems yield hepatocytes that are functionally limited, as shown by transient mitogenic responsiveness over a 2–3-wk period (3,13). Recent studies indicate that cell expansion and specific differentiation patterns can be stimulated in cultured cells with a defined, growth factor-enriched medium and extracellular matrix (3). Rat hepatocytes have been maintained for up to 2 mo. with a synthetic matrix of biodegradable polymeric scaffolds composed of polyglycolic acid (PGA) (4,14). These cells produced albumin (4) and expressed cytochrome P450 enzyme activity that was measured by the ability of cells to transform the nonfluorescent compound ethoxy-fluorescein ethyl ester to fluorescein (14). Hepatocyte cultures have also been maintained with an artificial capillary system and a hollow fiber bioreactor with cells entrapped in collagen (9,21). While previously used systems support spheroid formation, they are labor-intensive and useful within only a narrow range of cell density.

The advent of the Rotary Cell Culture System (RCCS), developed at the Johnson Space Center, Houston, Texas (20), has enabled the growth of suspension cells as well as anchorage-dependent cells in a simulated microgravity environment (2,7,10). This system simulates microgravity by randomizing the gravitational vector while supporting cellular colocation in three-dimensional space in a low shear, high mass transfer environment. In this study, we demonstrated that long-term cultures of PHLC can be maintained in a RCCS bioreactor under conditions of simulated microgravity. The morphologic and immunophenotypic properties of these have been characterized.

MATERIALS AND METHODS

Bioreactor. Synthecon Inc. (Houston, TX) kindly provided us with the RCCS bioreactor, which consists of a 50-ml vessel with oxygenator membrane made from silicon rubber. This membrane allows the oxygen to diffuse easily into the culture medium. Also, it facilitates gas exchange and keeps the level of pO_2 and pCO_2 stable in the medium. This vessel horizontally rotates about its axis by electric motor. Cells and pieces of liver tissues were maintained in suspension by balancing their sedimentation-induced gravity with centri-

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fugation caused by RCCS vessel rotation (20–30 rpm) with bubble-free oxygenation. All experiments with the bioreactor were performed in a humidified CO_2 standard incubator with 95% air and 5% CO_2, and constant atmosphere at a temperature of 37° C. The sampling and change of the media were performed under sterile conditions in a standard biological cabinet.

Microcarriers and scaffolds. Collagen-coated dextran beads, Cytodex 3 ($\Phi \sim 175$ μm) (Pharmacia Biotech, Inc., Uppsala, Sweden) and collagen-coated polystyrene beads ($\Phi \sim 90$–150 μm) (SolloHill Engineering, Inc., Ann Arbor, MI) that are used as microcarriers for cells were prepared and sterilized according to manufacturer's instructions. Polyglycolic acid (PGA) scaffolds (8) are biocompatible, biodegradable nonwoven fiber-based polymers, which were obtained from Albany International Research Co. (Mansfield, MA). Scaffolds were 5-mm-diameter and 2-mm-thick precut disks that were formed as a 97% porous mesh of 13-μm-diameter fibers. The scaffolds were packaged in double plastipeel pouches, sterilized with 8.6% ethylene oxide and 91.4% Freon at 130° F for 2 h and 10 min and aerated for 12 h. Before use, the scaffolds were washed in culture medium to remove contamination with toxic ethylene oxide. Ten scaffolds per bioreactor were used during each experiment. Fifty million cells in 5 ml culture medium were seeded onto scaffolds in a 50-cc Falcon conical tube for 30 min and gently stirred every 5 min in a 37° C humidified 5% CO_2 incubator. Following incubation, the cells with scaffolds were transferred into the RCCS and 45 ml of culture medium was added. A similar technique was used to seed cells on Cytodex-3 microcarriers. For a 50-ml cell suspension of 50 million cells, 2 g of Cytodex was added.

Cells and culture conditions. Human hepatoblastoma cells, HepG2, were obtained from the American Type Culture Collection and maintained in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO).

Liver segments were obtained through the Liver Tissue Procurement and Distribution system. Primary human hepatocytes (PHH) were harvested as previously described (1), with sequential perfusion of EDTA solution (Earle's balanced salt solution without Ca^{++} and Mg^{++}, plus 0.5 m*M* EDTA, 10 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) pH 7.4, and 1% gentamycin [GIBCO]) followed by collagenase solution (Earle's balanced salt solution with Ca^{++} and Mg^{++}, plus 0.5 mg collagenase P [Boehringer Mannheim Corp., Indianapolis, IN] per ml and 16 mg soybean trypsin inhibitor (Sigma, St. Louis, MO) per ml). Each solution was allowed to perfuse the organ for 15 min at a flow rate of about 100 ml/min at 37° C with a closed perfusion system through a blood warmer. After perfusion, the capsule was removed and hepatocytes were gently removed from the vascular tree by agitation in the collagenase solution at 37° C. Cells were placed into rinsing and plating medium (Eagle's minimum essential medium [MEM]/Weymouth's medium (3:1, tyrosine-free JRH, Lenexa, KS) plus 10% fetal bovine serum (FBS), 10 m*M* HEPES pH 7.4, and 1% gentamycin) and filtered through sterile gauze. To enrich the hepatocyte fraction, the cells were washed twice by low speed centrifugation (50 ×g). Cell viability was assessed by trypan blue exclusion. An identical number of cells (1×10^6 cells/ml in 50 ml RCCS) was seeded into the bioreactor in each experiment. Fresh medium was replenished by 80% of the total RCCS volume every 48 h or based on metabolic requirement, i.e., glucose level. PHH were cultured in a hormonally defined medium (SUM/CHOW: MEM/Weymouth's [3:1, tyrosine-free] plus 100 ng insulin per ml, 1 μg glucagon per ml, 50 ng epidermal growth factor per ml, 10 microunits somatotropin per ml, 20 milliunits prolactin per ml, 5 μg linoleic acid per ml, 3 n*M* selenium, HEPES (pH 7.4), 0.1 μ*M* dexamethasone, 1 μ*M* thyroxine, 10 μg transferrin per ml, and 20 ng Gly-His-Lys per ml). Glucose levels and urea formation in culture media were analyzed with the i-STAT Analyzer (i-STAT Corp., Princeton, NJ).

Primary human liver cell (PHLC) preparation included additional cellular components, i.e., fibroblasts and endothelial cells, Kupffer cells and bile duct epithelial cells, which we obtained from the mixed cellular population of the initial liver digest by omitting the centrifugation steps and by washing the mixed cells using gentle removal of perfusion solution following sedimentation of the cells. This mixed population of cells was resuspended in the same medium and cultured under the same conditions as PHH.

To assess the feasibility of culturing liver tissues obtained from surgical specimens or native livers removed at the time of transplantation, a portion of liver was gently minced into small pieces (1–3 mm), washed with medium, and cultured in the RCCS as described above.

Immunohistochemistry. Cells or tissues were fixed in 10% buffered formalin and embedded in paraffin as described (15). Paraffin-embedded thick sections were deparaffinized and then rehydrated in phosphate-buffered saline (PBS). The sections were stained with the Super Sensitive Kits (BioGenex, San Ramon, CA) according to the manufacturer's instructions. Monoclonal antibodies (MAb) against cytokeratins CK-7 (marker of bile duct epithelium) and CK-19 (marker of ductal and progenitor cells), proliferating cell nuclear antigen (PCNA), and CD-34 antigen (marker of endothelial cells) were obtained from BioGenex. MAb against E-cadherin was purchased from Transduction Labs (Lexington, KY). Apoptotic cells were identified with the TACOS-2 apoptosis kit (Trevigen, Inc., Gaithersburg, MD). Cell proliferation was assessed by 5-bromo-2'deoxyuridine (BrdU) incorporation into cellular DNA with the Labeling and Detection Kit-II (Boehringer Mannheim).

RNA and protein analyses. Levels of albumin (Alb) mRNA were assessed by Northern blot as described (15). Analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA served as an internal control. Albumin production was detected in serum- and albumin-free culture media by Helena serum protein electrophoresis (Helena Laboratories, Beaumont, TX) on cellulose acetate according to the manufacturer's instructions. As a control, we used normal human serum. Relative percent and absolute values for each band were calculated with The Helena CliniScan Densitometer with computer accessories.

Transmission electron microscopy. Ultrastructural cell morphology was examined by electron microscopy as follows. The cell samples were removed from the RCCS, washed in cold PBS and then fixed with 2% (vol/vol) glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.4, for 1 h at 4° C. The fixed cells were washed three times in cacodylate buffer, pH 7.2, containing 0.2 *M* sucrose, postfixed with 1% OsO_4 in 0.3 *M* cacodylate buffer, dehydrated with graded acetone and finally embedded in Epon-812. Ultrathin sections were stained with uranyl acetate and lead citrate and observed on a JEOL 1200 EX electron microscope at 80 kV. All reagents for EM were purchased from EMS, (Fort Washington, PA).

Scanning electron microscopy. The cell samples were removed from the RCCS, washed in cold PBS, and then fixed with 3% (vol/vol) glutaraldehyde and 2% paraformaldehyde in 0.1 *M* cacodylate buffer, pH 7.4, for 1 h at 4° C. Samples were prepared for scanning electron microscopy as described (12). Samples were scanned on a JEOL T330 electron microscope at 5 kV.

Results

Several approaches were used to examine the culture of human hepatoma (HepG2) cells and primary hepatocytes in RCCS bioreactors. These experiments evaluated the role of the extracellular matrix and other cellular components in the establishment of long-term cultures of functional hepatocytes. Moreover, we assessed the roles of microcarriers and biodegradable scaffolds in promoting growth, improving nutritional uptake, and facilitating oxygenation within cellular spheroids.

Analysis of HepG2 cultures in RCCS. HepG2 cells formed small spheroidal clusters 1–3 mm long within 48 h. These cells exhibited accelerated growth rates. Microscopic analysis revealed the presence of apoptotic centers in spheroids that exceeded 80–100 layers of cells in diameter or approximately 30 layers of cells or 200–400 μm from the periphery to the margins of apoptotic centers (Fig. 1 *a,b*). The proportion of apoptotic cells within the center of each spheroid increased with the size of the spheroids. Therefore, in small spheroids less than 1 mm in diameter, no apoptosis was observed. With growth, usually after 1 wk in culture, apoptotic cells were noted and their numbers increased accordingly to the size of the spheroid. This extent of layering is comparable to the size limit of tumor-cell populations that survive in vivo without blood vessels (6). Characterization of growth revealed that the majority of BrdU-positive cells were localized to the peripheral layers of spheroids, suggesting that cell proliferation occurs at the periphery. Therefore, cells would subsequently migrate into the intermediate zone and become quiescent. To address this hypothesis, we examined the expression of the cell adhesion molecule, E-cadherin. The expression of this protein occurs predominantly in differentiated cells and inversely correlates with

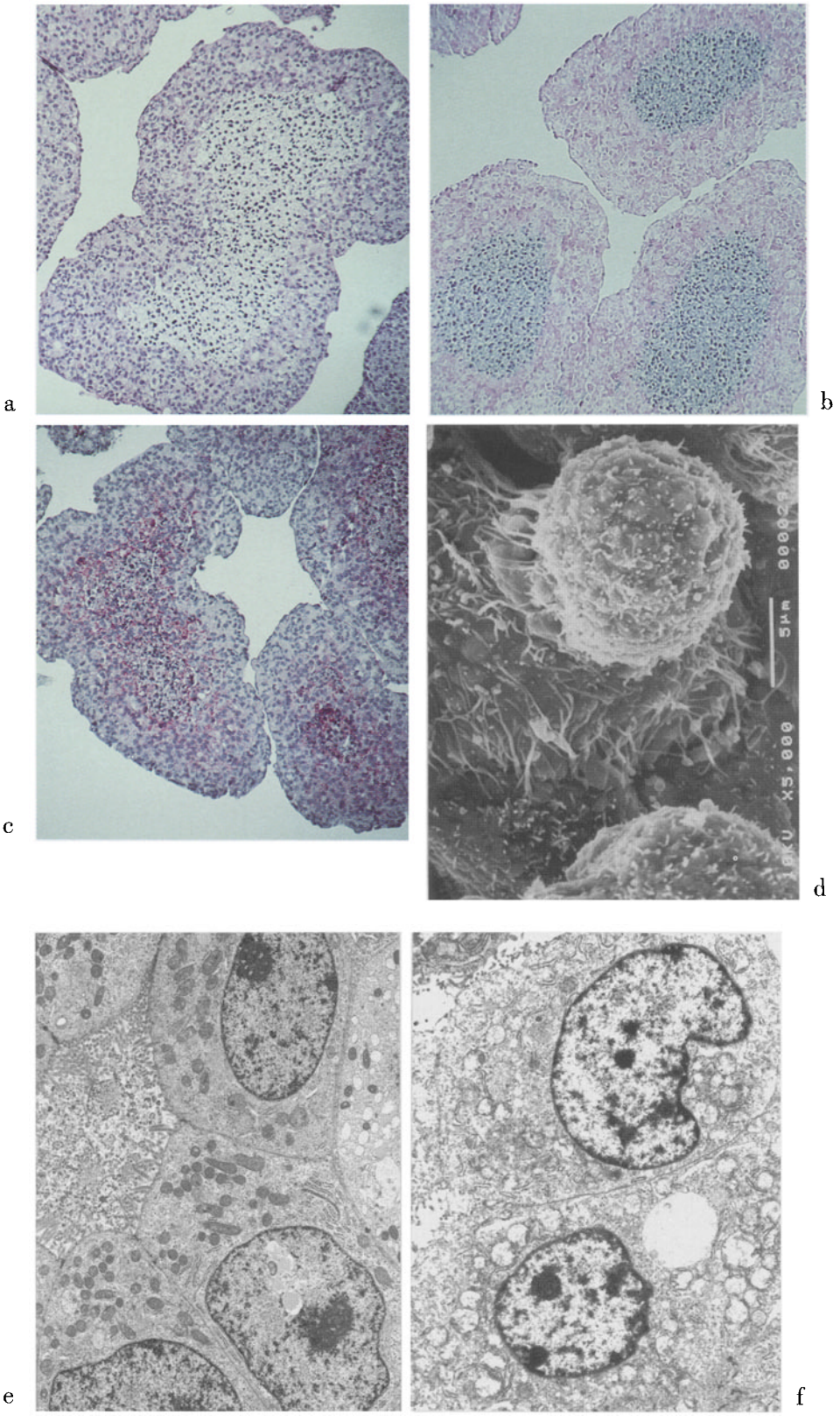


FIG. 1. Morphology and immunohistochemistry of HepG2 cells cultured in the RCCS. *a*, Histology (H/E stain) of HepG2 forming well-organized tight spheroids. *b*, Detection of apoptosis (blue stain) by in situ analysis. Apoptotic centers were present in large cell aggregates with diameter exceeding 30 cell layers. *c*, Immunohistochemical stain for E-cadherin (red color) in central and intermediate zones. Magnification, ×200. *d*, Scanning electron micrograph of Hep G2 cells after 7 d of culture. *e,f*, Transmission electron micrographs of cultured HepG2 cells after 7 d: (*e*) in bioreactor, (*f*) in conventional culture. Magnification, ×6000. Cells cultured in the RCCS developed tight junctions and well-formed bile canaliculus (BC).

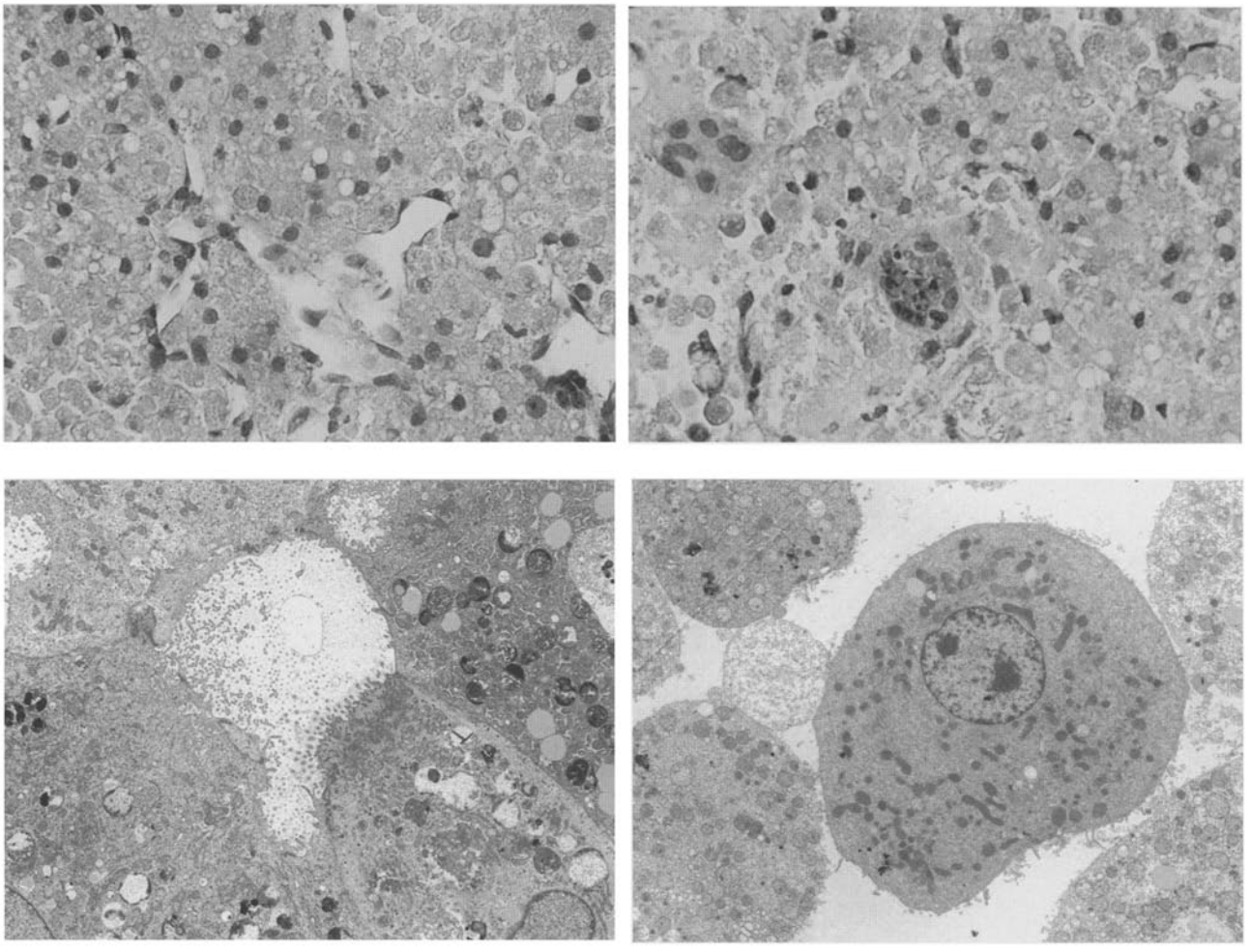


FIG. 2. Morphology of PHH cultured in the RCCS. *a,b,* Histology (H/E stain) of PHH obtained from a healthy donor at Day 15 in culture (×130). *a,* Cells are cohesive and form structures that are reminiscent of the hepatic architecture. An endothelial-cell lined vascular space is surrounded by viable hepatocytes arranged in plates. *b,* Hepatocytes and a tubular structure suggestive of a bile canaliculus. Some degenerating cells are seen in areas distant from vascular and biliary structures. *c,d,* Transmission electron micrographs of PHH: at Day 25 in culture in RCCS (*c*), before culturing (*d*). Magnification, ×3750. In contrast to features of hepatocytes before culture, the RCCS-cultured hepatocytes developed tight junctions and well-formed bile canaliculus (BC).

cell invasiveness (18). Immunostaining revealed that E-cadherin was primarily localized within the intermediate layers of spheroids, whereas staining was negligible at the periphery (Fig. 1 *c*). Therefore, this result supports the conclusion that the intermediate layers do not contain proliferating cells. Spheroids that were found to contain both proliferating and apoptotic cells exhibited a pattern similar to that observed in prevascular in situ carcinomas (6). These observations are consistent with the absence of angiogenesis as well as a diminished supply of oxygen and nutrients to these spheroids.

Since apoptosis was observed in the center of large cell aggregates, growth conditions were modified to improve oxygenation and nutritional uptake within these cells. When HepG2 cells were cultured with microcarriers, aggregates formed slowly and spheroids were reduced in size. Scanning EM of HepG2 cells grown in the presence of microcarriers (Cytodex-3) showed microvilli on the surface of a majority of cells (Fig. 1 *d*). This finding indicates that the hepatocytes were healthy and functionally active. Moreover, transmission EM of aggregates revealed a group of cells that were in the process of forming a bile canaliculus with multiple microvilli and tight junctions (Fig. 1 *e*). In contrast, no well-formed tight junctions or bile canaliculi were found in HepG2 cells cultured by conventional methods (Fig. 1 *f*).

Formation of cell aggregates and morphology of PHH cultures. The viability of PHH suspensions ranged from 87 to 94% according to trypan blue dye exclusion. Cell aggregates were collected and analyzed every 48 h. PHH cultures formed cell aggregates that were 2–4 mm long within 48 h. Light microscopy analysis of H&E staining of PHH aggregates demonstrated liver tissue-like structures that resembled poorly formed liver nodules with formation of primordial plates around a central space. Fig. 2 *a* and *b* shows the histology of the assembly of cultured PHH in RCCS. Cells were cohesive and formed structures clearly reminiscent of the poorly organized hepatic architecture. Transmission EM of PHH cultures revealed a loosely cohesive group of hepatocytes forming bile canaliculi with multiple

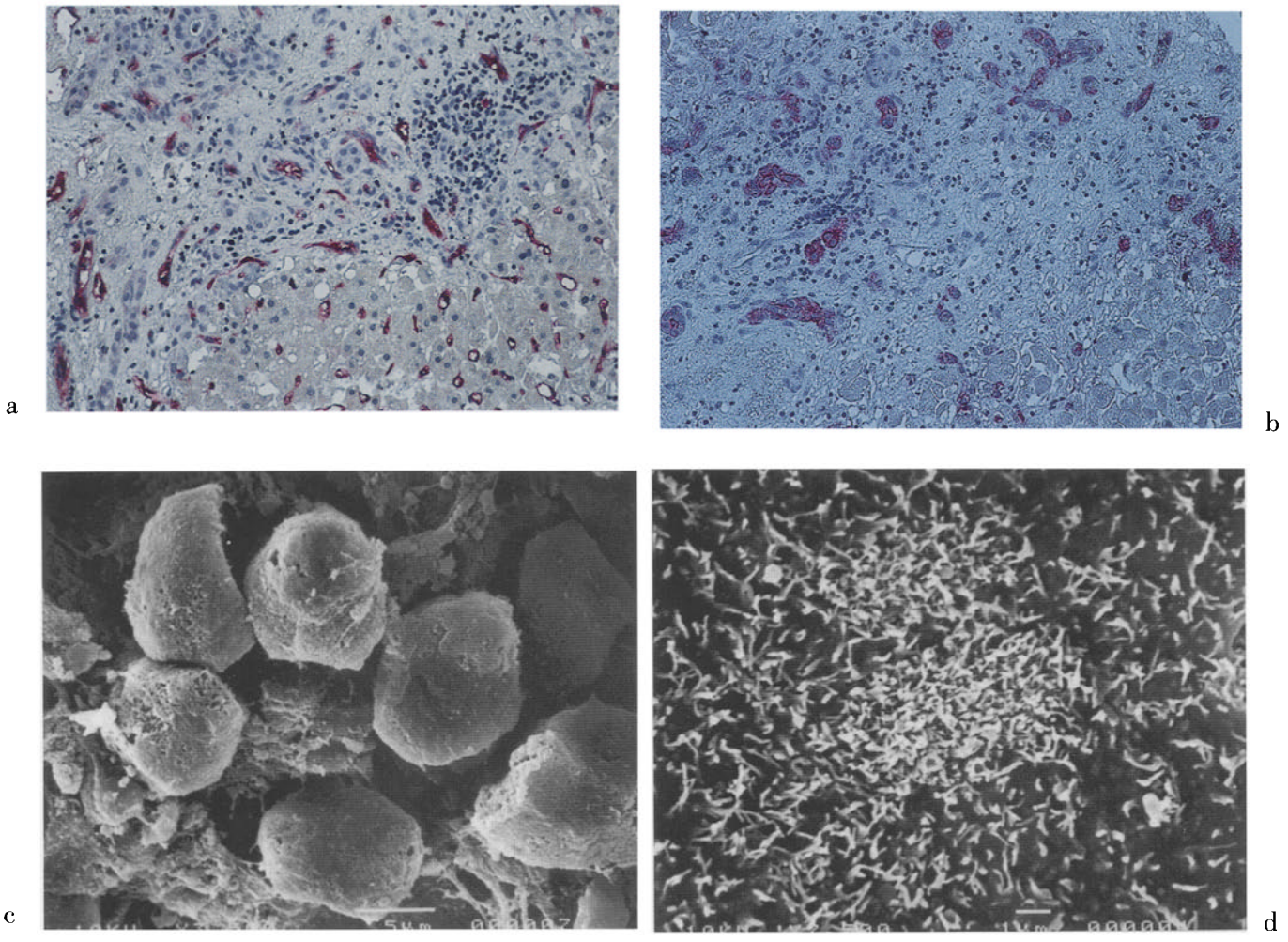


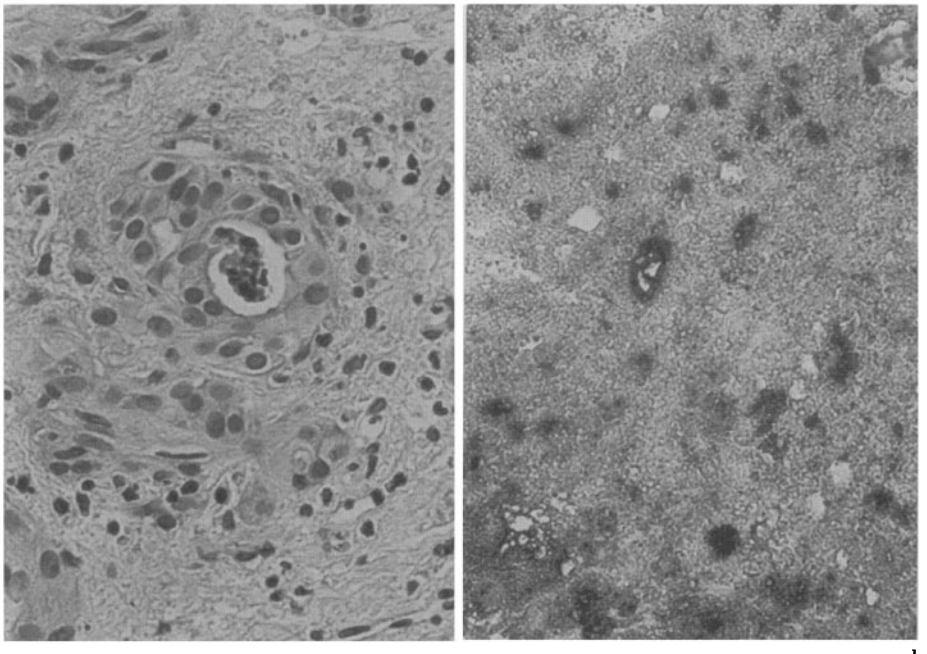
FIG. 3. Morphology and immunohistochemistry of PHLC cultured in the RCCS. *a,b,* A 15-d culture of PHLC. *a,* Cells stained with anti-CD34 monoclonal Ab (QBEnd/10), a marker of endothelial cells. Magnification, ×130. *b,* A 15-d culture stained with Cytokeratin 19 monoclonal Ab (RCK108), a marker of either bipotential progenitor cells in liver or biliary epithelial cells. Magnification, ×130. *c,d,* Scanning electron micrographs of cultured PHLC obtained from a normal liver (15 d in culture). *c,* Several rounded hepatocytes organized in tight clusters were surrounded by highly complex extracellular stromal structures and reticulin fibers. Magnification, ×1500. *d,* A higher power micrograph of hepatocytes clearly demonstrates microvilli along the sinusoidal surface of hepatocytes. Magnification, ×15 000.

microvilli and tight cellular junctions (Fig. 2 *c*). Moreover, the cytoplasm of the hepatocytes showed multiple lysosomes, lipid droplets and mitochondria. In contrast, EM of hepatocytes before culturing revealed only single hepatocytes with abundant cytoplasm, mitochondria, and only a few lipid droplets and foci of degeneration. No tight junctions or bile canaliculi were noted (Fig. 2 *d*). As with HepG2 cells, in PHH aggregates, the majority of apoptotic/necrotic cells were located in center. However, in contrast to HepG2, aggregates were not well organized to form rounded spheroids, and both apoptotic and necrotic cells were found throughout the aggregate. Therefore, the precise ratio or number of viable cells versus apoptotic/necrotic cells could not be estimated.

To confirm the viability of cells in aggregates, after 30 d in culture in the RCCS, some of the aggregates were transferred from the bioreactor and were subcultured in Falcon T-75 flasks. The cells from tissue-like structures or aggregates migrated onto the plastic and grew as a monolayer. However, within 1 wk of subculturing, cells began to detach from the plastic and exhibited apoptosis. Interestingly, this behavior was similar to that observed when freshly isolated hepatocytes (PHH) were cultured on plastic flasks.

Formation of liver tissue-like structures and ultrastructural organization of PHLC and minced liver tissue. PHLC cultures formed spheroids that were 2–5 mm long within 48 h. These spheroids increased to 1 cm long within 1 wk. Macroscopic examination indicated the presence of a three-dimensional tissue-like assembly. Subsequent analyses were carried out over a period of 60 d. We monitored cell viability and growth by measuring glucose use and urea formation.

The histology of PHLC spheroids after 10 d in culture revealed morphological evidence of proliferating epithelial cells that formed bile duct-like structures. In addition, we observed endothelial cells with vascular sprouts. Immunohistochemical examination confirmed



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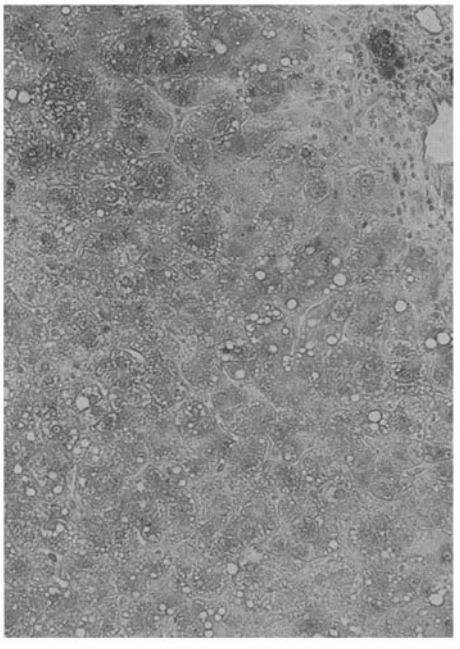

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FIG. 4. Morphology and immunohistochemistry of minced liver tissue cultured in the RCCS. *a*, Histology (H/E stain) of minced liver tissue at Day 10 in culture. Magnification, × 130. *Proliferating epithelial cells are* arranged to form a bile duct-like structure. Endothelial cells are organized in vascular sprouts. A loose matrix representing fibrous tissue surrounds these structures. Magnification, ×130. *b,c,* Determination of cell proliferation by BrdU incorporation. *b,* Minced liver tissue cultured for 15 d in a bioreactor and labeled with BrdU 24 h before immunostaining. *BrdU incorporation* into cells (deep blue stain). *c,* Staining of unlabeled liver (control) specimen. Magnification, ×120.

the expansion of endothelial cells, bipotential progenitor liver cells, and/or biliary epithelial cells by Day 15 in the bioreactor (Fig. 3 *a,b*). Transmission EM of cultured PHLC revealed that cohesive groups of hepatocytes formed bile canaliculi with multiple microvilli and tight cellular junctions, as observed with PHH cultures. Scanning EM of PHLC cultured for 15 d showed clusters of rounded hepatocytes with *microvilli along* the sinusoidal surface (Fig. 3 *c,d*). Occasional endothelial-like cells displaying sieve plates were observed in sinusoidal-like structures. It is of interest that in cultures of primary liver cells, proliferating epithelial cells that formed bile duct-like structures and viable hepatocytes were more abundant in the vicinity of newly formed vascular sprouts than elsewhere (Fig. 3 *a*).

We also cultured liver cells from minced liver tissue (1–3 mm^3). Throughout the 30 d of these cultures, the liver fragments maintained tissue-like structural organization. Within 15–30 d in the bioreactor, these cultures contained bipotential progenitor liver cells and endothelial and/or biliary epithelial cells. Moreover, as observed in vivo during vigorous liver regeneration, numerous bile duct-like structures were formed (Fig. 4 *a*). Consequent collagen formation, which was determined with Trichrome stain (Sigma Chemical Co., St. Louis,

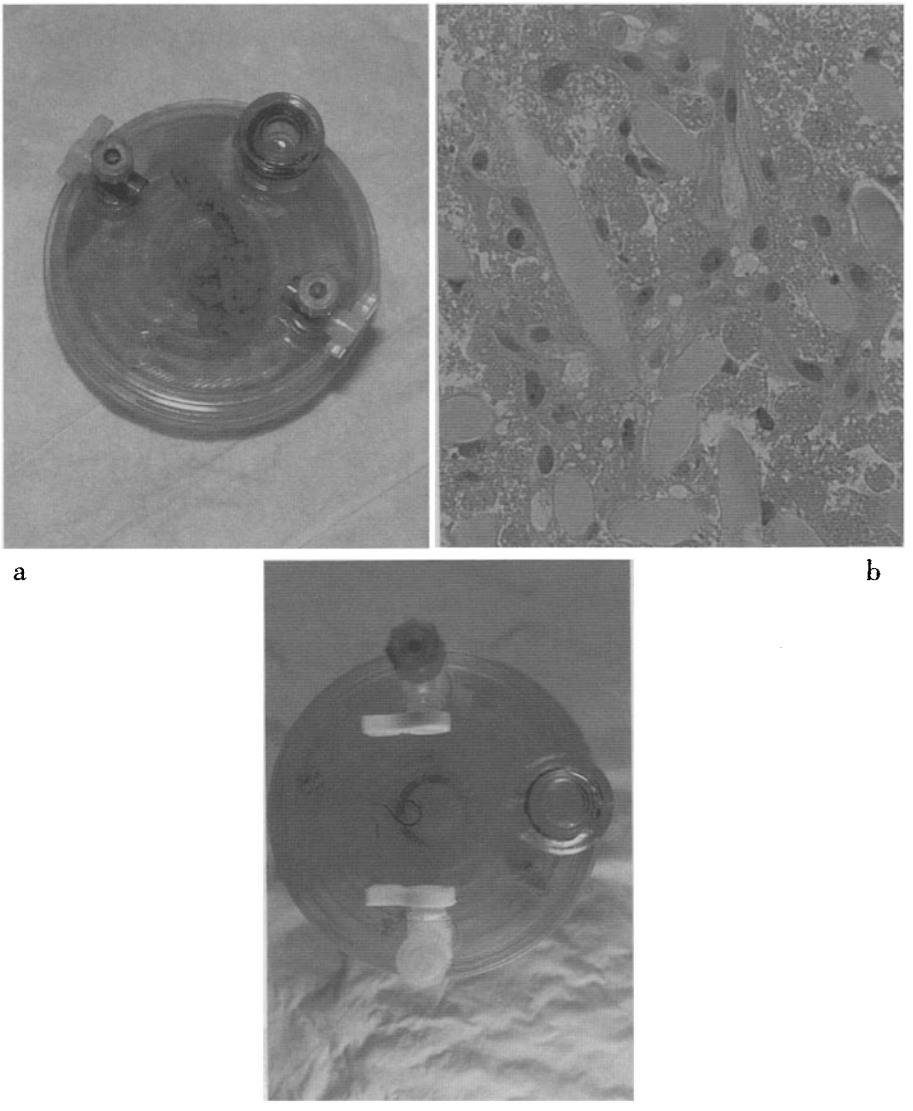

FIG. 5. PHLC cultured with and without PGA scaffolds. *a,c,* Macroview of cell aggregates in RCCS after 7 d in culture. PHLC cultured with scaffolds is shown in panel *a* and without polyglycolic (PGA) scaffolds in panel *c*. *b,* H/E stain of PHLC with PGA scaffolds demonstrates cell distribution between the fibers of the scaffolds. Magnification, ×400. This sample was obtained from the large aggregate of PHLC that is shown in panel *a*.

MO), was observed during the progress of the culture period (data not shown). We assessed cell proliferation and DNA synthesis by examining BrdU incorporation into cellular DNA. These studies revealed 10 to 15% BrdU-positive liver cells at Day 15 of the cultures (Fig. 4 *b*), which correlated with PCNA expression in cell spheroids (data not shown). Therefore, these data suggest that minced liver tissue can be effectively cultured in the RCCS.

Analysis of PHH and PHLC cultures with microcarriers and biodegradable scaffolds. We proceeded to analyze cultures grown in the presence of collagen-coated beads such as microcarriers and PGA scaffolds. These agents were used to improve the overall oxygenation and nutritional uptake within the center of PHH and PHLC spheroids. As we indicated, the histology of large cell aggregates of primary hepatocytes cultured without microcarriers revealed apoptotic/necrotic centers similar to those found in HepG2 cultures. The use of microcarriers prevented the formation of apoptotic or necrotic zones in these aggregates. However, aggregates formed slowly and were reduced in size (1–2 mm with Cytodex; 1–3 mm with SoloHill).

Synthetic polymers can stimulate isolated cells to regenerate tissue with defined sizes and shapes and are currently being studied as scaffolds for cell transplantation both in vitro and in vivo. Polyglycolic acid (PGA) is a synthetic polymer that permits cell adhesion and growth without provoking inflammation or toxicity when implanted in vivo. PGA provides a processable frame of three-dimensional structural material, which provides a high surface area for cell–polymer interaction with minimal diffusional constraints during in vitro culture (8). Culturing PHH and PHLC in the presence of PGA scaffolds resulted in more efficient cell assembly and the formation of larger aggregates. After 1 wk in culture, these cells (PHLC) formed aggregates up to 3 cm long (Fig. 5 *a*). The mechanisms of promoting formation of large aggregates remain unclear. It is conceivable that both passive entrapments with attachments of hepatocytes to scaffold as well as subsequent aggregation of several scaffolds occur during long-term culture in simulated microgravity. Fig. 5 *a* demonstrates an ensemble of several scaffolds with attached hepatocytes in one large aggregate. The histology of aggregates cul-

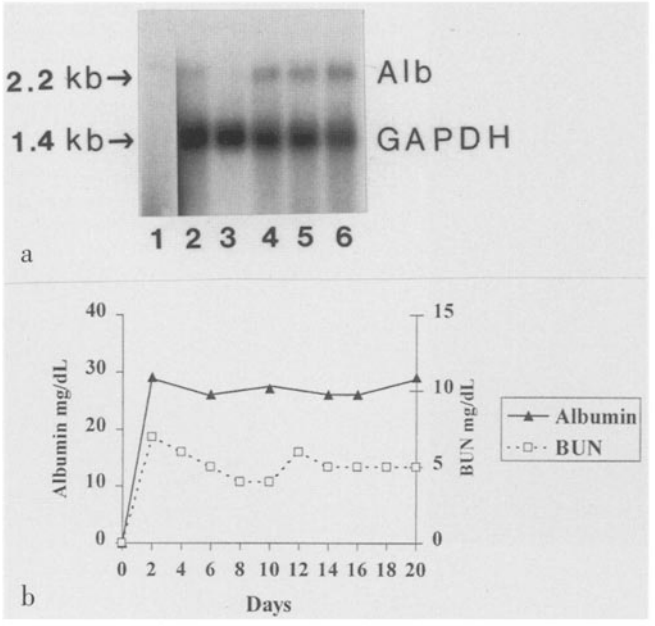

FIG. 6. *a*, Northern blot analysis of albumin gene expression in PHLC during 60 d in culture. Five micrograms of total RNA from PHLC aggregates was hybridized to DNA probes for human albumin and GAPDH genes. Exposure time was 72 h. Lines: (1) 0 d, (2) 24 d, (3) 30 d, (4) 38 d, (5) 44 d, and (6) 60 d of RCCS culture PHLC. *b*, Albumin secretion and BUN production in PHLC cultured in the RCCS.

tured with PGA showed polymer fibers with attached hepatocytes (Fig. 5 *b*). As a control, Fig. 5 *c* shows formation of cell aggregates from the same PHLC that were cultured without scaffolds.

Albumin mRNA expression, albumin production, glucose utilization and urea formation. To assess the functional activity of hepatocytes cultured in the bioreactor, we carried out a time-course analysis of albumin mRNA expression. Albumin mRNA was detected throughout the 60-d period, with moderately reduced levels during the first few days. Representative results derived from long-term PHLC cultures are shown in Fig. 6 *a*.

In selected experiments we also analyzed cell culture medium for albumin production. Using Helena serum electrophoresis methodology on cellulose acetate, we evaluated production of albumin and globulin fractions in culture media. Secretion of albumin and alpha-1 globulin fraction was demonstrated by clearly visible protein bands through the duration of the experiments. Estimated albumin production during a 20-d period was in the range of 26 to 29 mg/dL/24 h (Fig. 6 *b*).

We monitored cell viability and the requirement for media replenishment by evaluating glucose use and urea formation in culture media that were collected every 48 h at the time of media replacement. Urea nitrogen measurements were consistently higher than 5 mg/dL (Fig. 6 *b*) and glucose utilization was approximately 50% of the original concentration in 48 h-spent media samples. These results indicate that hepatocytes cultured in the simulated microgravity environment were metabolically active during the 60-d period. In contrast, primary hepatocytes cultured on plastic surfaces in the presence of gravitational forces failed to produce urea and albumin after 5 to 7 d in culture.

DISCUSSION

Our results with primary and neoplastic hepatocytes indicate that the microgravity environment created by the RCCS is conducive to cell aggregation and the formation of liver tissue-like structures. Cultures of HepG2, PHH, and PHLC formed spheroids within 48 h. The formation of spheroids has been shown to promote liver-specific functions (8). When compared to HepG2 and PHH spheroids, PHLC spheroids formed more rapidly and continuously grew. The pronounced growth of PHLC in this system emphasizes the importance of the extracellular matrix and other liver cell components. Histological and immunohistochemical analyses of PHLC spheroids revealed multidimensional liver tissue-like structures. These structures were formed by hepatocytes, proliferating biliary epithelial cells, and/or progenitor liver cells. Furthermore, liver-like structures were observed that resembled bile ducts arranged along nascent vascular spaces.

Cells cultured in bioreactors in the presence of microcarriers exhibited improved nutrient and waste transfer. These cultures were reduced in their capacity to form apoptotic centers. However, cell aggregates formed slowly and were small. It is conceivable that when the microcarrier beads are covered with cells, cells and beads raft together and form large dense cellular packs with compromised oxygenation in the centers. This process may ultimately lead to disintegration of large cell aggregates. In contrast, culturing cells with biodegradable PGA scaffolds increased the efficiency of cell self-assembly and the formation of larger cell aggregates. On the basis of histological evaluation, it appears that the degree of necrotic/apoptotic cells was dramatically diminished as compared to cultures without scaffolds. These results are consistent with the recent observations suggesting that PGA scaffolds stimulate isolated cartilage cells to regenerate tissue in defined sizes and shapes (7,12). Furthermore, in the RCCS, gas exchange and oxygen supply is provided by passive diffusion through an oxygenator membrane that affords controlled supplies of oxygen and nutrients with minimal turbulence and extremely low shear. Similar membranes are used in heart-lung machines. Differentiated cells requiring rapid transport of nutrients associate closely with endothelial cells of adjacent blood vessels (kidney) or sinusoids (liver). The fine structures of liver sinusoids implicate crucial interactions between hepatocytes and the endothelium. Hepatocytes are in contact with the single layer of endothelium that lines the liver sinusoids and is the primary barrier between the hepatocytes and blood. Indeed, coculture of rat hepatocytes with sinusoidal cells (endothelium and Kupffer cells) resulted in longer survival of functional and differentiated hepatocytes (19). Therefore, significant expansion of liver mass or the remodeling of liver in vitro will be implausible without optimizing oxygenation and nutritional uptake within growing cellular aggregates. Our cultures of PHLC or minced liver have exhibited proliferating biliary epithelium and the formation of a new vascular supply. Therefore, the expansion of in vivo-like tissue structures is favored when the perfusion of nutrients and oxygen is improved via de novo formation of capillaries (16). This process is facilitated by cocultures containing cellular components that are critical for formation of angiogenic vascular structures within the growing tissue mass.

The mechanisms responsible for cell self-assembly, spheroid formation, and the development of extracellular matrix in bioreactors remain to be established. A combination of factors are likely to play important roles in the formation of tissue-like structures. These in-

clude systems for artificial hepatic support and hepatocyte transplantation. Progress in these areas has been constrained by the inability to establish the "ideal" hepatocyte in culture. The ideal hepatocyte should be of human origin, normal (nonmalignant) phenotype, readily available, and rapidly and easily grown in cell culture; should remain stable in a well-differentiated state for days to weeks; and should be capable of the full range of synthetic and detoxifying features of mature hepatocytes (11). Previous work has failed to establish a human hepatocyte culture with these properties. Therefore, alternative research models have used primary hepatocytes from other mammalian species (pig, rat, and dog) and immortalized human liver cell lines.

We have demonstrated the feasibility of culturing PHLC in a simulated microgravity environment within bioreactors for an extended period of time. By establishing a long-term culture system for large masses of functioning PHLC, our work may facilitate the development of improved protocols for autologous hepatocyte transplantation, gene therapy, and liver-assist devices. It is conceivable that with further improvements, the RCCS bioreactor will be used to produce simulated liver tissue with defined size and shape as shown with chondrocytes (7). Therefore, this system may facilitate efforts to implant ex vivo-grown tissue-like structures. This process may permit the correction of metabolic defects by implanting expanded tissue that has been genetically manipulated in vitro. Also, tissue-like structures produced in a simulated microgravity environment could be evaluated with respect to three-dimensional cell-to-cell interactions. Finally, this system can be used to investigate regulatory molecules that control cellular differentiation and transformation in normal and neoplastic tissues, respectively.

ACKNOWLEDGMENTS

This work was supported by NASA grants NAG5-3712, NAG8-1383 and Texas Medical Center, Houston, NASA/Johnson Space Center Cooperative Agreement NCC 9-36.

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