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EXTENDED LIVER-SPECIFIC FUNCTIONS OF PORCINE HEPATOCYTE SPHEROIDS ENTRAPPED IN COLLAGEN GEL

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The potential use of porcine hepatocytes in a bioartificial liver device requires large quantities of viable and highly active cells. To facilitate the scaling up of the system, liver specific activities of hepatocytes should be maximized. One way of enhancing the specific activities is to cultivate hepatocytes as multicellular spheroids. Freshly isolated porcine hepatocytes form spheroids when cultivated in suspended cultures. These spheroids exhibit higher activities for a number of liver specific functions compared to hepatocytes cultivated as monolayers. However, these activities decreased in a few days in culture. Entrappment of spheroids in collagen gel sustained their metabolic activities at a stable level over 21 days. Production of albumin and urea by spheroid hepatocytes entrapped in collagen gels were 2 to 3 times higher than those by freshly isolated single cells. P-450 activity was demonstrated by metabolism of lidocaine to its main metabolite, monoethylglycinexylidide. Phase II drug metabolism was demonstrated by glucuronidation of 4-methylumbelliferone. This work shows that porcine hepatocyte spheroids entrapped in collagen maintain differentiated functions for an extended time period. Such hepatocyte spheroid entrappment system may facilitate the development of a bioartificial liver support device.

Key words: porcine hepatocyte; spheroids; P-450; glucuronidation; artificial liver.

INTRODUCTION

A collagen gel entrapment hollow fiber bioreactor employing xenogeneic hepatocytes has been developed as an extracorporeal bioartificial liver (BAL) system for providing short-term support to patients in hepatic failure. Rat hepatocytes entrapped in a BAL device have been shown to be metabolically active and exhibit a number of liver functions (23,24) in tests employing animal models (25). A BAL system with porcine hepatocytes has recently been scaled up for evaluation in a large animal model of D-galactosamine induced liver failure (35). However, the device needs to be further scaled up for human application in the treatment of hepatic failure.

Recent developments in cell culture technology have sustained long-term survival and maintained hepatocyte differentiated functions. These methods include cultivation of hepatocytes as spheroids (16,37) and collagen gel immobilization (8,15,31). Hepatocytes grown in a spheroid culture system reorganize themselves into three-dimensional cyto-architecture similar to that observed in vivo (2,17,22). In such structures, cell to cell contacts are maximized, and the hepatocytes survived longer while maintaining their liver-specific functions (11). We have developed a stirred culture method for generating large quantities of porcine spheroids in a short time (19). Morphologic studies showed that the spheroids maintain many of the cyto-architecture characteristics found in vivo, such as junctional complexes, canaliculus-like structures, and microvilli.

The possible use of spheroids in a BAL system requires demonstration of multiple biological functions performed by the liver. This includes P-450 biotransformation (phase I activity) and conjugation (phase II activity). Other major liver functions reported to be maintained at high levels in spheroids include albumin synthesis (21,38), tyrosine aminotransferase activity (18,37), alpha-fetoprotein activity (37), urea synthesis (12,36), ammonia release (36), and glutamic-pyruvic transaminase (GPT) activity (36). In this report, three typical liver metabolic functions, ureagenesis, albumin production, and drug biotransformation, were investigated using spheroids of porcine hepatocytes entrapped in collagen. Drug biotransformation was measured with marker substrates for phase I (lidocaine) and phase II (4-MU) activity. It is hypothesized that the use of entrapped spheroids instead of dispersed hepatocytes within the BAL, could lead to significant improvement in function and facilitate the potential human trials.

MATERIALS AND METHODS

Hepatocyte Preparation

Hepatocytes were harvested from 6- to 10-kg male pigs (Midwest Research Swine, Gibbon, Minnesota) by a two-step in situ collagenase perfusion technique modified from the method described by Seglen (34). The animal was anesthetized with a combination of ketamine (100 mg/ml): rompun (100 mg/ml), 5 ml:1ml i.m., then intubated and mechanically ventilated. Anesthesia was maintained with isoflurane (1.5%) via the endotracheal tube, and muscle contraction was paralyzed with succinylcholine (20 mg i.v.). The abdomen was entered through a bilateral subcostal chevron incision. The venous vascular supply to and from the liver was completely isolated and looped with

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ties. The hepatic artery, common bile duct, gastrohepatic omentum, and phrenic veins were ligated. The portal vein was cannulated with tubing, and perfusion was initiated at 300 ml/min with oxygenated perfusion solution I (Per I). Per I consisted of 143 m*M* sodium chloride, 6.7 m*M* potassium chloride, 10 m*M* hydroxyethylpiperazine-ethanesulfonic acid (HEPES), and 0.1% ethylene glycol-bis-aminoethyl ether (EGTA), pH 7.4. The suprahepatic and infrahepatic vena cavae were ligated and a vent was made in the infrahepatic cava in order to modulate perfusion back pressure. The liver was excised, placed in a large sterile basin, and perfused at 300 ml/min with perfusion solution II (Per II). Per II consisted of 100 m*M* HEPES, 67 m*M* sodium chloride, 6.7 m*M* potassium chloride, 4.8 m*M* calcium chloride, 1% bovine albumin, and 0.1% collagenase-D (Sigma, St. Louis, MO), pH 7.6. After 20 to 30 min there was visual and palpable evidence of the liver dissolving. The liver capsule was then broken and the liver substance was raked and irrigated with cold William's E medium (GIBCO, Grand Island, NY) supplemented with 15 m*M* HEPES, 0.2 U/ml insulin (Eli Lilly, Indianapolis, IN), 2 m*M* L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The released cells were filtered through 100-μm nylon mesh and resuspended with fresh William's E medium. Viability was assessed by trypan blue exclusion (28).

Hepatocyte Culture

The isolated hepatocytes, having a viability of above 90%, were resuspended in hormonally defined culture medium designated LTE at a concentration of 5×10^5 cells/ml. The LTE medium was a modification of the serum-free medium of Enat et al. (9) and contained William's E basal medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 0.2 U/ml insulin (Eli Lilly), 1 μ*M*/ml dexamethasone (Sigma), 4 ng/ml glucagon (Sigma), 25 μg/ml EGF (Sigma), 20 ng/ml liver growth factor (Sigma), 6.25 μg/ml transferrin (Sigma), 50 ng/ml linoleic acid (Sigma), 500 μg/ml albumin (Sigma), 0.1 μ*M* copper ($CuSO_45H_2O$), 3 n*M* selenium (H_2SeO_3), 50 p*M* zinc ($ZnSO_47H_2O$), and 15 m*M* HEPES (GIBCO), pH 7.4.

Monolayer cultures. Freshly isolated hepatocyte suspension (5×10^5 cells/ml) in LTE medium was used to inoculate 12-well tissue culture polystyrene plates (Falcon Multiwell, Becton Dickinson, Franklin Lakes, NJ) at 2 ml/well. Culture medium was replaced daily with fresh medium, the spent medium was frozen at −20° C until assayed.

Spheroid formation in spinner flask. Spheroid formation was carried out by inoculating 100 ml of hepatocyte at a concentration of 5×10^5 cells/ml in LTE medium into spinner flasks stirred at 80 rpm in an incubator maintaining a temperature of 37° C and a highly humidified atmosphere of 95% air:5% CO_2. The medium was changed 24 h after cell inoculation and every 48 h thereafter by centrifugation (30 g for 3 min) by fresh medium.

Collagen entrapment. Freshly isolated hepatocyte suspension or 24-h-old spheroids were suspended in a mixture (3:1 vol/vol) of type I collagen solution (Vitrogen 100, Celtrix Santa Clara, CA) and fourfold concentrated Williams' E medium supplemented with 0.8 U/ml insulin, 400 U/ml penicillin, and 400 μg/ml streptomycin sulfate at pH 7.4. The cell concentration was 5×10^5 cells/ml. The collagen-cell suspension was then inoculated into 12-well plates at 1 ml/well and incubated at 37° C for 15 to 20 min to accelerate gel formation. Subsequently, 2 ml of LTE medium was added and the medium was changed daily. The spent medium was stored at −20° C before analysis. The rate of gel contraction by the entrapped cells was measured using these disk-shaped collagen gels. The diameter of gels, initially 20 mm, was measured daily using a ruler as described previously for measuring collagen contraction by fibroblasts (5) and recombinant human 293 cells (32).

Scanning Electron Microscopy (SEM)

Spheroids from 5-day-old culture were fixed with 4% glutaraldehyde in 0.2 *M* sodium cacodylate buffer (pH 7.2) for 90 min, followed by 2% osmium tetraoxide (OsO_4) for 30 min. The cells were dehydrated through ascending concentrations of ethanol and dried in a critical point dryer and then coated with gold in an Sputter Coater (EMS 76M, Ernest Fullham, NY). The coated samples were observed under a scanning electron microscope (Hitachi, Japan).

Urea and Protein Assays

Urea levels were determined by the Urea Nitrogen Diagnostic Kit (Sigma, procedure no. 640), according to the methods described by Fawcett and Scott (10) and Chaney and Marbach (6). Urea production rates by immobilized cultures (collagen-entrapped and monolayer) were calculated from urea levels in a 24-h culture supernatant divided by the inoculated cell number. Production rates of spheroid cultures was calculated from urea levels in cell-free supernatants and cell number determined from the protein content of the culture (0.5 mg protein is equivalent to 1×10^6 cells). Total protein was determined by the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce Chemical Company, product no. 23225), using bovine serum albumin as a standard.

Albumin Determination

Porcine albumin concentrations were determined by a sandwich enzyme-linked immunoassay (ELISA). Briefly, Maxisorp plates (GIBCO BRL) were coated with 1:1000 rabbit antiserum to porcine albumin (Cappel, Durham, NC) in pH 9.4 carbonate buffer. The plates were incubated overnight at 4° C, washed with 0.05% Tween 20 (Bio-Rad, Hercules, CA) in phosphate buffered saline (PBS), blocked for 2 h at 37° C with 0.5% gelatin in PBS and rewashed. Serial dilutions (1:4) of unknown samples and 10 μg/ml porcine albumin standards (Sigma) were added (100 μl/well). The plates were incubated for 1 h at 37° C and rewashed. One hundred microliter per well of peroxidase-conjugated goat anti-swine albumin (Bethyl Labs, Montgomery, TX) diluted 1:16 000 in Tween/PBS were added, the plates incubated for 1 h at 37° C and rewashed. The plates were developed with 100 μl/well of 55 mg/ml 2,2′Azino-di(3-ethylbenzthiazoline-6-sulfonate) (Boehringer-Mannheim, Indianapolis, IN) for 1 h at room temperature, then read at 405 to 490 nm using an ELISA reader (Bio-Tek, El-312e, Winooski, VT). Unknown concentrations were obtained by performing a four parameter logistic regression on the optical densities generated from the dilutions of the standard; determining a relative dilution factor for each unknown by fitting each unknown's optical densities to the logistic regression equation of the standard; and calculating the actual concentration from the standard concentration and the relative dilution factor for each unknown. Specific productivity was determined as described for urea production.

Drug Metabolism

To evaluate P-450 and glucuronidation activities, the medium from 12-well plate cultures of monolayer or collagen-disk was withdrawn and replaced with equal volume of medium containing 12 μg/ml lidocaine (Abbott Laboratories, Chicago, IL), and 65 μ*M* H-methylumbelliferone (4-MU) (Sigma). The medium of the spinner flask

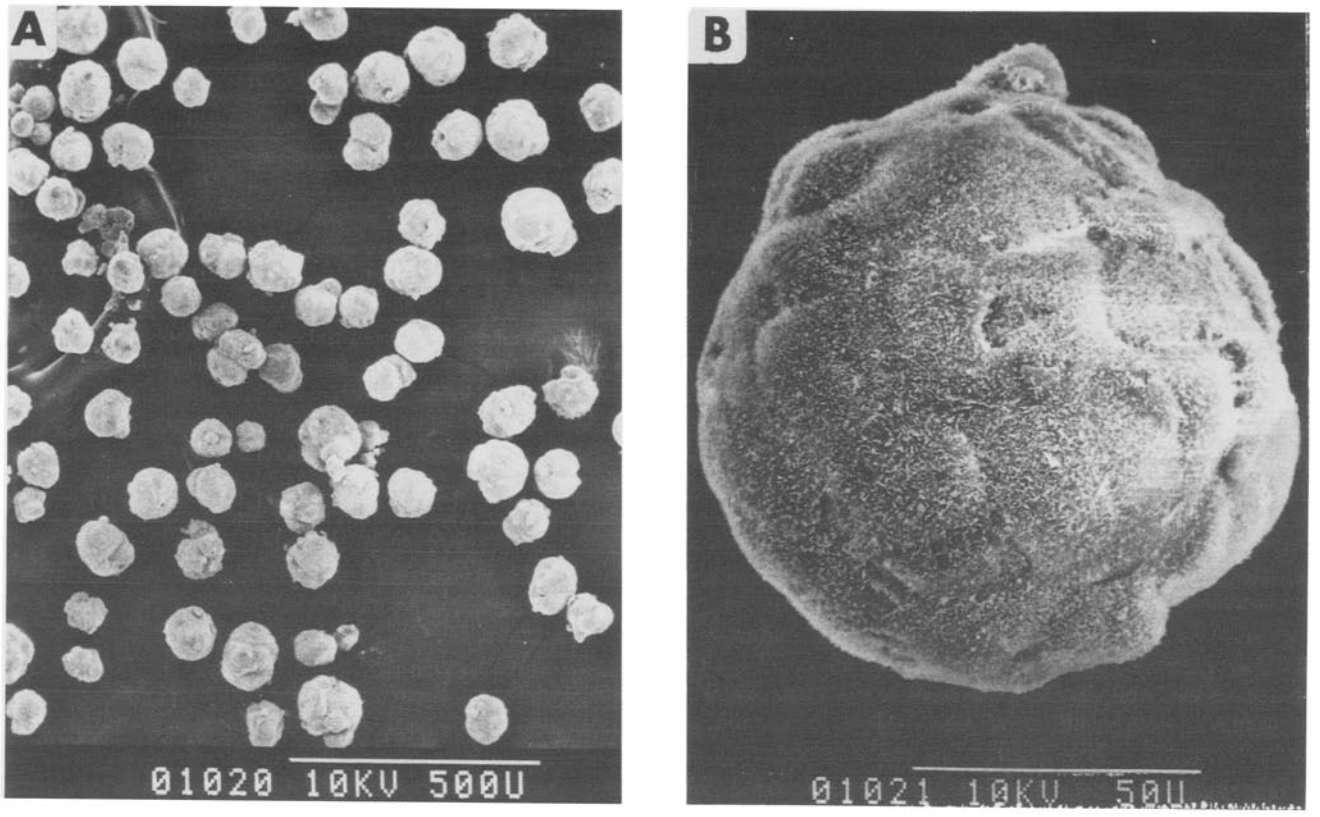

FIG. 1. Scanning electron micrographs of porcine hepatocytes spheroids. *A*, low magnification showing a relatively uniform spheroid shape; *B*, high magnification showing extensive cell-to-cell contact, numerous microvilli, and some holes on the spheroid surface.

culture of spheroids was similarly replaced except that the content of the flask was centrifuged at 30 g for 3 min to remove the spent medium completely before being resuspended in lidocaine and 4-MU-containing medium. The medium was replaced daily afterward and the spent medium was stored frozen until analyzed for drug biotransformation. Lidocaine and its main metabolite monoethylglycinexylidide (MEGX) were analyzed by gas chromatography with nitrogen-phosphorus detection (25). 4-MU and 4-MUG were detected by high performance liquid chromatography (HPLC) according to the procedure of Lovdahl et al. (20).

RESULTS

Spheroid Formation

Hepatocytes cultured in stirred conditions form multicellular spheroids within 24 h. These spheroids exhibit compact morphology and smooth, undulating boundaries. During that 24-h period, cells first agglomerated to form aggregates of irregular shape and "bumpy" boundaries. Subsequent rearrangement and compaction of cells gave rise to relatively smooth, spherical boundaries. By 24 h, almost all aggregated cells were in the form of spheroids. Spheroids grew in size from 40 to 70 μm in diameter in the 1st day to 100 to 140 μm after five days in culture. Scanning electron microscopy showed that spheroids were relatively uniform in size and spherical in shape (Fig. 1 *A*). At a higher magnification, extensive cell-to-cell contact, numerous microvilli, and small holes, apparently corresponding to bile canaliculi structures, can be seen on the surface of the spheroid (Fig. 1 *B*).

Gel Contraction

The rate of gel contraction by both spheroids and dispersed hepatocytes entrapped in collagen was measured with disk-shaped gels as described in Materials and Methods. The spheroids were obtained from 24-h spinner culture whereas the dispersed cells were used immediately after hepatocyte harvest. Spheroids were prepared from hepatocytes harvested from the same pig as those of dispersed cells. The initial time points of contraction measurements of the two cases were thus 24 h apart. The cell concentration in collagen gel in both cases was 1×10^6 cells/ml. As a control, hepatocytes were killed by exposing to 50% ethanol for 15 min before collagen entrapment. Each data point in Fig. 2 represents the average of the major and the minor axis measurements. These results demonstrate that viable hepatocytes are required for collagen gel contraction. The rate of gel contraction by hepatocytes entrapped as spheroids seemed to be higher than that by hepatocytes entrapped as single cells. The contraction is affected by cell concentration (24,32) as well as the distribution of cells in the gel. The distribution of spheroids and dispersed cells in the gel is likely to be different, this could be attributed to the difference in the contraction rate. The observation of gel contraction is important to the application of spheroids in the BAL system because gel contraction is necessary for intraluminal perfusion

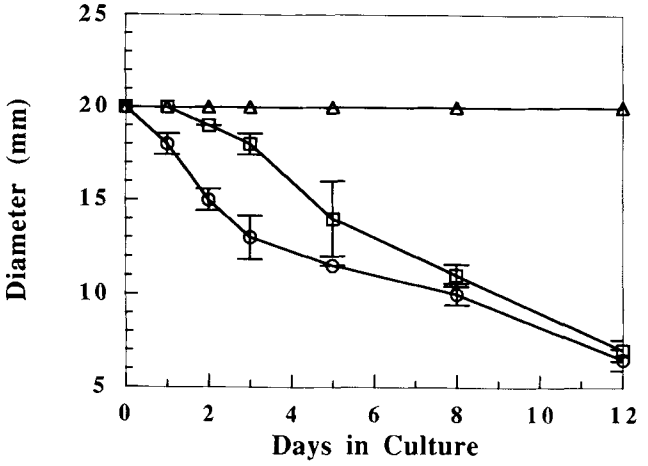

FIG. 2. Contraction of collagen gel disks containing entrapped single-cell hepatocytes, hepatocyte-spheroids, and dead hepatocytes killed by incubation in 50% ethanol for 15 min at room temperature. Each point is the average diameter (± standard deviation) of three gels. Initial hepatocyte concentration in each gel was 1×10^6 cells/ml. (*Open circles*) spheroids; (*open squares*) dispersed cells, (*Triangle*) dead cells.

of culture medium to the hepatocytes entrapped in a hollow fiber bioreactor.

Ureagenesis

Urea production by hepatocytes cultivated as monolayer and as spheroids was compared. The number of hepatocytes inoculated per unit volume of medium was the same in both cases. After urea concentration measurements the daily production of urea was calculated. The rate of urea production divided by the initial hepatocyte concentration was taken as the specific urea production rate. Figure 3 *A* presents urea production over a period of 21 days by hepatocytes cultivated as suspended spheroids or as monolayer cultures. Specific productivity in both cultures increased for the first three days and then gradually decreased. Hepatocytes cultured as free spheroids were two times more active than cells grown as monolayer on tissue culture plates.

Both singly dispersed cells and spheroids were also entrapped in collagen gel disks at the same cell concentration, submerged in medium, and cultivated in 12-well plates. The urea production was compared (Fig. 3 *B*). Again urea production was higher in the spheroid culture after collagen entrapment. Furthermore, the urea production by both collagen-entrapped spheroids and dispersed cells was more stable than those in free suspension or as monolayer. After initial increase in activity in the first three days, the urea production rate decreased to a level similar to Day 1 and remained little changed until Day 21. Entrapment of spheroids and unaggregated cells in collagen gels thus extended this synthetic activity.

Albumin Production

Albumin synthesis by spheroids in spinner flasks and cells cultivated as monolayer was measured and expressed as cumulative values over 7 days (Fig. 4). Specific albumin synthesis rates were determined by a linear regression fit of the data. The albumin pro-

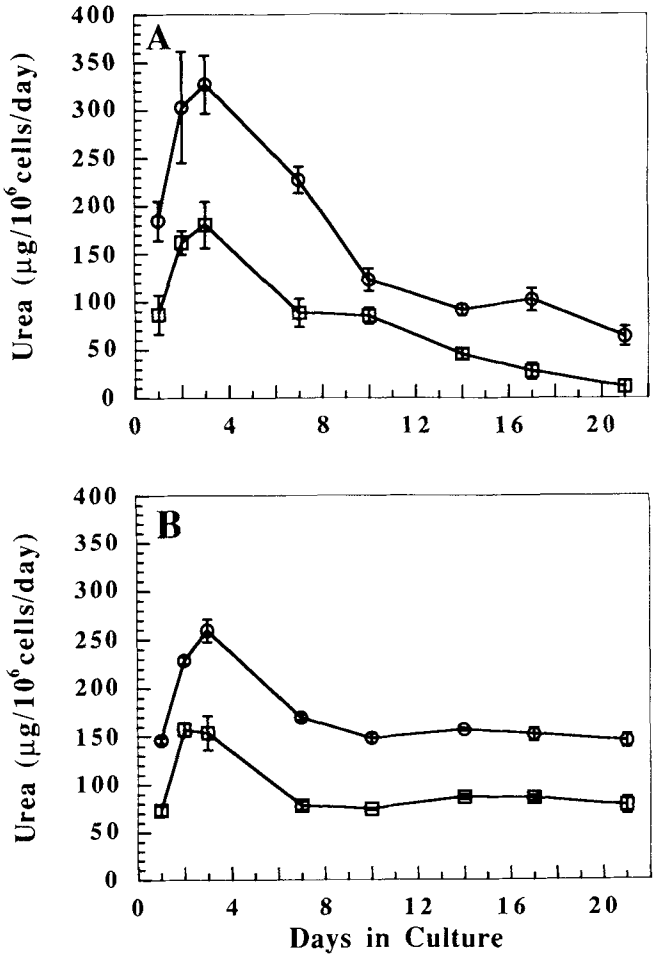

FIG. 3. Specific urea production by porcine hepatocytes cultivated as dispersed cells and spheroids. *A*, stirred spheroids and monolayer hepatocyte cultures, (*open circles*) spheroids, (*open squares*) monolayer; *B*, collagen-entrapped spheroids and dispersed cells, (*open circles*) spheroids, (*open squares*) dispersed cells.

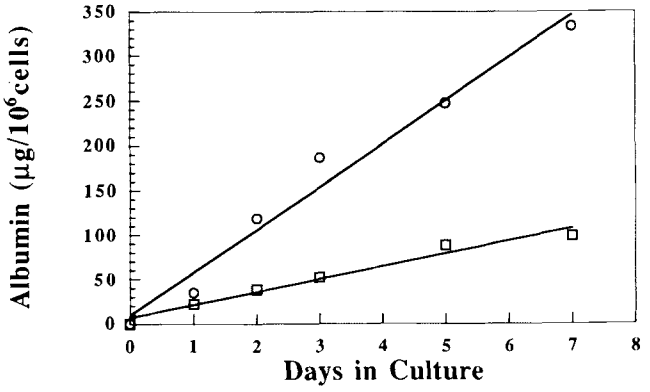

FIG. 4. Kinetics of albumin production by free-suspended spheroids and unaggregated cells cultivated as monolayers on tissue culture plates; (*open circles*) spheroids, (*open squares*) monolayer.

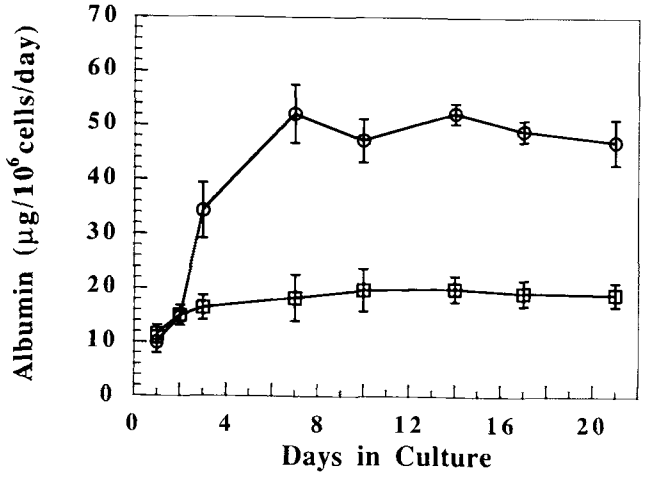

FIG. 5. Specific albumin production by collagen-entrapped spheroids and unaggregated hepatocytes; (*open circles*) spheroids; (*open squares*) dispersed cells.

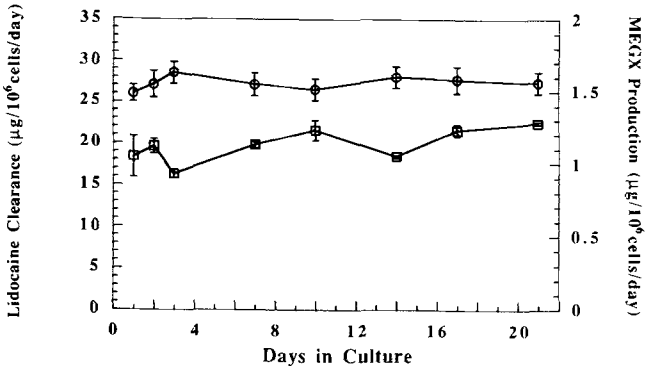

FIG. 6. Lidocaine clearance rate and MEGX formation in collagen-entrapped spheroid culture. (*open circles*) lidocaine clearance; (*open squares*) MEGX formation.

duction rate for spheroid and monolayer cultures were determined to be 50 μg/10⁶ cells/day and 14 μg/10⁶ cells/day, respectively.

The albumin production by collagen-entrapped spheroids and dispersed hepatocytes was measured for 21 days. The specific activities were calculated and are shown in Fig. 5. After an initial period of increasing activity, they stabilized at approximately 50 $\mu g \cdot 10^6$ $cells^{-1} \cdot day^{-1}$ and 18 $\mu g \cdot 10^6$ $cells^{-1} \cdot day^{-1}$, respectively. The albumin synthetic activity was not significantly changed from that in free-suspension or monolayer and was maintained for at least 21 days. This production level is similar to the in vivo albumin production reported for human liver (29).

Lidocaine Metabolism

The cytochrome P-450 function of hepatocyte spheroids entrapped in collagen was evaluated by lidocaine metabolism (Fig. 6). Both the disappearance of exogenously added lidocaine and the appearance of the metabolic product, MEGX, were measured. Lidocaine clearance alone is not a sufficient representation of P-450 activity because the drug might be taken up by the hepatocytes without further biotransformation. Thus, production of lidocaine metabolites, e.g., MEGX, was also measured to validate the quantitation of P-450. Lidocaine clearance remained relatively constant at about 28 $\mu g \cdot 10^6$ $cells^{-1} \cdot day^{-1}$ over the 21-day period. MEGX specific production was also maintained relatively constant at a rate of approximately 1.2 $\mu g \cdot 10^6$ $cells^{-1} \cdot day^{-1}$, demonstrating the continued function of the cytochrome P-450 enzyme system.

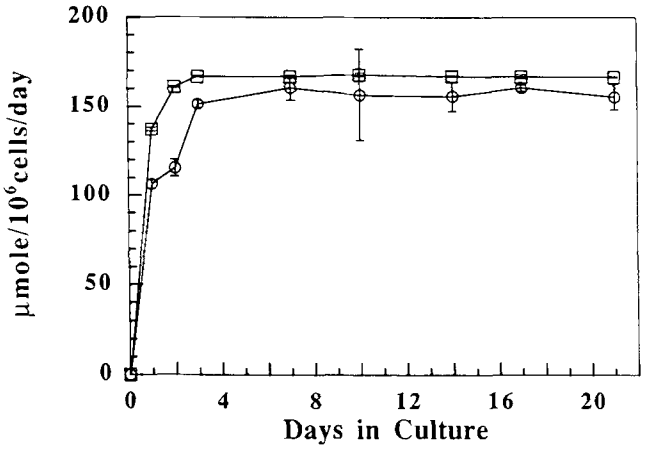

FIG. 7. 4-MU clearance rate and glucuronidation of porcine spheroids entrapped in collagen gel. (*open circles*) 4-MU clearance; (*open squares*) 4-MUG formation.

4-MU Conjugation

The conjugational activity of hepatocyte spheroids entrapped in collagen gel was examined by 4-MU conjugation. 4-MU concentration decreased from 65 μ*M* to below 0.1 μ*M* within 24 h. The glucuronidated metabolite, 4-MUG, appeared in the culture medium. The specific rates of 4-MU disappearance and 4-MUG appearance are shown in Fig. 7. High glucuronidation activity was maintained in culture throughout the 21-day period. We could not detect the sulfated 4-MU metabolite (4-MUS) at a sensitivity of 1 μ*M*. This activity presents the capability of pig hepatocyte spheroids to carry out phase II metabolism for long time periods while entrapped in collagen gel.

DISCUSSION

The use of hepatocyte spheroids in an extracorporeal BAL system has been suggested by several investigators (16,37) because spheroids exhibit improved cell viability and enhanced specific liver functions. Entrapment of hepatocytes in collagen gels supports long-term differentiated function of the cells (15). In this study we showed that cultivation of porcine hepatocyte spheroids in collagen gels can be a useful in vitro model for studying hepatocyte function in long-term cultures in a potential BAL system.

Demonstration of liver function is essential for the potential application of the spheroids in a BAL system. We evaluated urea production and albumin synthesis as an indication of liver-specific synthetic functions and lidocaine and 4-MU metabolism as an affirmation for drug metabolism. Essentially all urea formed in the human body is synthesized in the liver. In the absence of the liver or in serious liver disease, ammonia accumulates in the blood, caus-

ing the development of encephalopathy (7). Urea production by freely suspended spheroids in stirred culture was twice as high as seen in single hepatocytes cultivated as monolayer cultures. The decline in ureagenesis during cultivation in both spheroid and monolayer cultures may be attributed to a decrease in cell activities with time; however, loss of viability and washout of cells during medium change may contribute to this phenomenon. When these cells were entrapped in collagen gels, the urea producing capability was extended for at least 21 days in culture. The decrease, if any, of cell viability with time did not significantly affect ureagenesis in the collagen-entrapped cultures.

Another important liver function is the production of albumin. Albumin was secreted by collagen-entrapped spheroids at a stable rate of 2 $pg \cdot cell^{-1} \cdot h^{-1}$ which corresponds to the in vivo albumin production by human liver (29). Production continued at a constant rate for at least 21 days. Non-entrapped cells cultivated as monolayer or free suspended spheroids lost significant albumin productivity after 7 days in culture (data not presented).

Lidocaine clearance has been proposed as a sensitive measure of hepatic function. Lidocaine is an important marker of drug metabolism, both as a model substrate of phase I (oxidation, reduction, and hydrolysis) metabolism (26,30) and as a clinical index of hepatic failure (33). Biotransformation of lidocaine has been shown to require the activity of multiple P-450 isoenzymes (1,3,13) located within the endoplasmic reticulum of the hepatocytes. A primary metabolite of lidocaine, MEGX, is formed via oxidative N-deethylation (27) and could also be used in the evaluation of liver function (39). P-450 activity has been suggested to be the critical function that must be provided by a successful bioartificial liver (14). Both lidocaine clearance and MEGX formation were maintained in collagen-entrapped spheroid cultures for at least 21 days, demonstrating stable P-450 activity by gel-entrapped spheroids.

Glucuronidation and sulfation of 4-MU, a phenol derivative, were measured to assess the conjugation (phase II) activity of the cells. These conjugation pathways are critical in the elimination of endogenous steroids and phenol molecules which have been associated with neurotoxic and comagenic effects[4]. The conjugation of 4-MU takes place within the cell; glucuronidation of 4-MU to 4-MUG is catalyzed by one of multiple isoenzymes of UDP-glucuronosyltransferase (UDPGT) located on the inner membrane of the endoplasmic reticulum. Sulfation of 4-MU to 4-MUS is catalyzed by sulfotransferase (ST), a cytosolic enzyme; this enzymatic activity was not detected by porcine hepatocyte cultivate either as single or spheroid cultures. The significance of this observation in a BAL system has to be studied.

Porcine hepatocyte spheroids entrapped in collagen are capable of phase I and phase II drug metabolism for a long time. They also exhibit high urea and albumin synthetic activities. The high and stable liver specific functions make the spheroid-collagen system attractive for further development in a BAL assist system. Investigation of a BAL employing such spheroids and randomized large animal trials are currently underway.

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