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Biochemical and Functional Changes of Rat Liver Spheroids During Spheroid Formation and Maintenance in Culture: I. Morphological Maturation and Kinetic Changes of Energy Metabolism, Albumin Synthesis, and Activities of Some Enzymes

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Abstract In the process of isolated single liver cells coming together to form three-dimensional spheroids, cells undergo dramatic environmental changes. How liver cells respond to these changes has not been well studied before. This study characterized the functional and biochemical changes during liver spheroid formation and maintenance. Spheroids were prepared in 6-well plates from freshly isolated liver cells from male Sprague rats by a gyrotatory-mediated method. Morphological formation, and functional and biochemical parameters of liver spheroids were evaluated over a period of 21 days in culture. Liver spheroid formation was divided into two stages, immature (1–5 days) and mature (>5 days), according to their size and shape, and changes in their functionality. Galactose and pyruvate consumption was maintained at a relatively stable level throughout the period of observation. However, glucose secretion and cellular GPT and GOT activities were higher in immature spheroids, decreased upto day 5 and remained stable thereafter. Cellular γ-glutamyltransferase (γ-GT) and lactate dehydrogenase (LDH) activities were initially undetectable or low and increased as spheroids matured. Albumin secretion decreased rapidly within the first 2 days and increased as spheroids matured. It is concluded that cells undergo functional and biochemical changes during spheroid formation following isolation of liver cells from intact tissue. Functionality and biochemical properties recovered and were maintained in mature spheroids. A relatively stable period (6–15 days) of functionality in mature spheroids was identified and is recommended for applications of the model. J. Cell. Biochem. 90: 1166–1175, 2003.

Key words: liver spheroid; GPT; albumin

Liver spheroid culture is a process that brings together single cells to form three-dimensional, multicellular aggregates. During this process, cells undergo significant environmental changes. The cells in spheroids resume cell–cell contacts, and survive and maintain liver-specific functions much longer and superior to cells cultured as monolayers [Landry et al., 1985; Lin et al., 1995; Lazar et al., 1995a; Juillerat et al., 1997; Hansen et al., 1998; Tzanakakis et al., 2001]. Liver spheroid culture, reported as early as the 1980s [Landry et al., 1985], was mainly employed in artificial liver studies [Lazar et al., 1995b; Hu et al., 1997]. It also showed potential applications in many other areas such as hepatotoxicology [Sipes et al., 1987; Dilworth et al., 2000; Walker et al., 2000], drug metabolism [Nakazawa et al., 1997], heat shock responses [Gosnell et al., 2002], and tumour studies [Roberts and Soames, 1993].

Several liver spheroid culture methods have been developed. These include self-assembly [Peshwa et al., 1994; Hansen et al., 1998; Yamada et al., 2001], magnetic bar rotation-mediated [Yagi et al., 1993], gyrotatory-mediated [Walker et al., 2000], spinner-mediated [Sakai et al., 1996], and foam pore-trapping [Iijima et al., 1998] methods, and entrapping in

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collagen gel [Hansen et al., 1998] or alginated beads [Khalil et al., 2001]. Whatever the culture method, the liver cells in preparing three-dimensional spheroid cultures experience a common process: intact liver tissue → single cells → spheroids. Thus spheroids are artificially formed, biologically-active spheres that are inherently different from other traditional in vitro models such as monolayer and liver slice cultures. Although some morphological aspects and functions of liver spheroids including urea and albumin syntheses and P450 activity have been reported [Niwa et al., 1996], many other liver specific functions and biochemical properties of liver spheroids have not been well studied previously. Understanding the biochemical characteristics of liver spheroids and their changes during spheroid maturation and maintenance are essential to promote the application of this model and its appropriate use.

In this study (Part I), we investigated the changes in energy metabolism, albumin synthesis, and the activities of some enzymes during spheroid preparation, maturation, and maintenance. Part II details nitric oxide (NO) synthesis and related changes in spheroids.

MATERIALS AND METHODS

Chemicals

L-Glutamine was obtained from GibcoBril, (UK). Fetal calf serum (FCS), hepatocyte medium, penicillin and streptomycin sulfate, galactose, and other chemicals and reagents were obtained from Sigma (UK).

Spheroid Culture

Liver cells were isolated from the liver of male Sprague rats (Charles River) by a collagenase perfusion method described by Vonk et al. [1978]. The cells were not purified and hence included all cell types in the liver. Viability was determined by trypan blue dye exclusion. Only isolated liver cell suspensions with a viability of 80% or over were used for spheroid culture. Spheroids were grown in 6-well plates in culture in hepatocyte medium supplemented with 5% FCS, 200 nM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 2 mg/ml galactose. This medium contains no glucose. The plates were incubated at 37°C on a gyrotatory-shaker placed within a 5% CO_2 incubator.

Morphological Observations

Morphological changes from single cells to mature spheroids were observed under light microscopy over 21 days. A mature spheroid was examined under Scanning Electron Microscopy (S-450, Hitachi Scientific Instruments, UK) after fixation in 2% glutaraldehyde in PBS buffer.

Ultra-structure of cells in liver spheroids was also observed under transmission electron microscopy (TCM). Liver spheroids were fixed in 4% glutaraldehyde in PBS for 1 h, post-fixed in 1% osmium tetraoxide for 1 h, and stained in 1% uranyl acetate for 1 h. Spheroids were sliced in polymerized Spurrs resin and observed under the TCM (CM10, Philips, Cambridge, UK).

Histological sections were stained by haemotoxylin and eosin [Wilson and Gamble, 2002].

Functional and Biochemical Evaluations

Functional and biochemical evaluations were studied at the time-points of 1, 2, 3, 6, 9, 12, 15, 18, and 21 days, respectively. One plate (6-wells) of liver spheroids was used for each time-point. Old culture medium from each well was completely replaced with serum-free medium 24 h before sampling at each time-point. The recipe of the serum-free medium was the same as culture medium but without FBS. Samples were collected from each time-point 24 h after changing the medium. Medium and spheroid samples were collected separately. Spheroids in each well were homogenized at 4°C in 1 ml homogenizing buffer containing 2 mM $NaH_2PO_4 \cdot 2H_2O$, 2 mM Na_2HPO_4, 0.5 mM EDTA, and 145 mM NaCl.

Biochemical Assays

Protein. Total protein was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Cat. no. 600-0005). Homogenates of 10 μl were added to each well of a 96-well plate (Greiner Bio-One, UK) followed by 200 μl of diluted protein reagent. Absorbance was read in a microplate reader (Multiscan RC, Labsystems, UK) at 595 nm.

Glucose secretion. Glucose in the medium was determined by a Glucose kit (Cat. no. 510-A, Sigma). Briefly, 50 μl of glucose standard or medium was added to each well of a 96-well plate followed by 250 μl of color reagent (PGO); each sample was run in duplicate. The plate was incubated at 37°C for 30 min and absorbance read in a microplate reader at 450 nm.

Galactose assay. Galactose in the media was determined by an enzymatic method described by Xu et al. [2002].

Albumin assay. Rat albumin in the culture media was determined with a rat albumin ELISA quantitation kit (Cat. no. E110-125, Bethyl Laboratories, Inc., UK). Each medium sample was diluted at 1:100 with sample diluent solution. The enzyme substrate reaction time was 20 min. Other detailed procedures followed the manufacturer's instructions. Absorbance was read in a microplate reader at 450 nm.

Pyruvate assay. Pyruvate was determined by a colorimetric method developed by Friedemann and Haugen [1943] and modified in our laboratory. Briefly, 10 μl of either standard or medium sample was added to each well of a 96-well plate followed by 40 μl distilled water and 50 μl 2,4-dinitrophenylhydrazine solution (0.2 mg/ml in 1 N HCl). The plate was left to stand at room temperature for 20 min. After that, 200 μl of 0.5 N NaOH was added and samples incubated at room temperature for 5 min. Absorbance was read at 492 nm in a microplate reader.

Cellular lactate dehydrogenase (LDH) activity. LDH activity in spheroids was determined using a lactate dehydrogenase kit (Sigma, Cat. no. 500-C), based on a colorimetric method developed by Cabaud and Wroblewski [1958]. Briefly, spheroid homogenate was diluted with homogenate buffer (1:1). Each sample of 10 μl was added to each well of a 96-well plate followed by 50 μl of 0.75 mM sodium pyruvate containing 1 mg/ml NADH to each well (not including the wells for the standard curve) and incubated at 37°C for 30 min; samples were run in duplicate. A 50 μl aliquot of 2,4-dinitrophenylhydrazine was added to each well and allowed to stand at room temperature for 20 min. Then 50 μl of 4 M NaOH was added to each well and allowed to stand for 5 min to develop the final color. Absorbance was read at 540 nm in a microplate reader.

Cellular γ-glutamyltransferase (γ-GT) activity. γ-GT activity in spheroids was determined using a γ-glutamyltransferase kit (Sigma, Procedure no. 545). Spheroid homogenate of 25 μl of each sample was added to each well of a 96-well plate, in duplicate. Following procedures refers to Procedure 454 (Sigma). Absorbance was read at 540 nm in a microplate reader.

Cellular alanine aminotransferase (GPT, ALT) activity. GPT was determined with an alanine aminotransferase (ALT/GPT) kit (Sigma, Cat. no. 505-P). Spheroid homogenate of 10 μl of each sample was added to each well of a 96-well plate, in duplicate, followed by 50 μl GPT substrate solution. After 1 h incubation at 37°C, 50 μl of color reagent was added to each well and the plate incubated for 20 min at room temperature. After incubation, 200 μl 0.5 N NaOH was added and absorbance read at 492 nm with a plate reader 5 min after NaOH.

Cellular aspartate aminotransferase (GOT, AST) activity. GOT was determined with an aspartate aminotransferase (AST/GOT) kit (Sigma, Cat. no. 505 AST/GOT). Spheroid homogenate of 10 μl of each sample was added to each well of a 96-well plate, in duplicate, followed by 50 μl GOT substrate solution. The rest of the procedure was the same as the GPT assay (see above).

Statistical Analysis

Student's t-test was used for comparisons and $P < 0.05$ was accepted as significant.

RESULTS

Morphological Maturity of Liver Spheroids

Under gyrotatory-mediated culture conditions, single liver cells initially aggregated together to form small cell aggregates within a few hours after plating (Fig. 1A). The small aggregates joined together to form larger aggregates as shown in Figure 1B–C. Gradually, aggregates increased in size and became more regular as shown in Figure 1D. This process normally took 4–5 days. Individual differences in spheroid formation from different rats was observed. Cells from younger rats (body weight <200 g) tended to form neat, regular spheroids (mature) earlier (at day 4) and cells from older rats (body weight >350 g) tended to form mature spheroids later (at day 5 or 6). So day 6 is a clear time-point for morphological maturation of liver spheroids. After this time, the shape and size of spheroids were relatively stable. Thus day 6 spheroids and thereafter were morphologically classified as 'mature' spheroids. Their size was 200 ± 25 μm in diameter. Accordingly, 1–5 days spheroids were classified as 'immature' spheroids.

Figure 2 shows the SEM image and histology of mature spheroids (6 days). As shown in

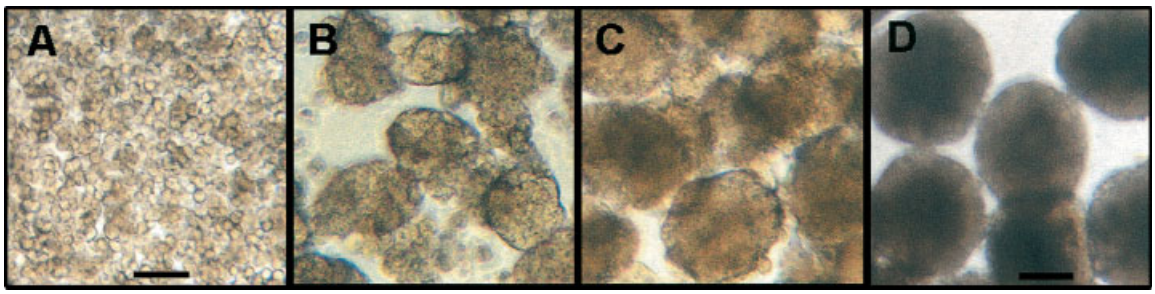

Fig. 1. The kinetics of liver spheroid formation. **A**: Single liver cells and small reaggregates, 2 h after plating; the bar represents 50 μm; **B**: Irregular aggregates, 24 h after plating. **C**: Irregular aggregates, 3 days after plating. **D**: Mature spheroids on day 6; the bar represents 100 μm (B, C, and D are on the same scale).

Figure 2A, the surface of the mature spheroid is relatively smooth and cell–cell contacts are tight. The histological architecture of a 6-day-old spheroid in transverse section shows that the cells in the spheroid resumed cell–cell contacts and their nuclei were distributed well over the section (Fig. 2B). The cells in the center are in a good condition and no necrotic centers were seen.

High power SEM shows that cell–cell contacts have been established, however, the boundary is not tightly sealed. There are "canals" which open to the surface (Fig. 3A). The internal ultra-structure shows a good contact between cells and the gap at a cell junction (Fig. 3B). Hinge-like cell–cell contacts can be occasionally seen (Fig. 3B). Details of some organelles can be seen as well, that indicate cells in a normal status.

Galactose Consumption

The spheroid culture medium contained 2.9 mg/ml galactose (the hepatocyte medium itself contained $0.9 + 2$ mg/ml of supplemented galactose) but no glucose. Liver spheroids retained the function of galactose metabolism. Galactose consumption by liver spheroids during spheroid formation and maintenance was relatively stable over the 21 days observation period (Fig. 4A). There was no significant difference between immature and mature spheroids in their consumption of galactose.

Pyruvate Consumption

Pyruvate consumption is shown in Figure 4B. Similar to galactose consumption, pyruvate consumption was relatively stable over the 21 days culture period. There was no significant

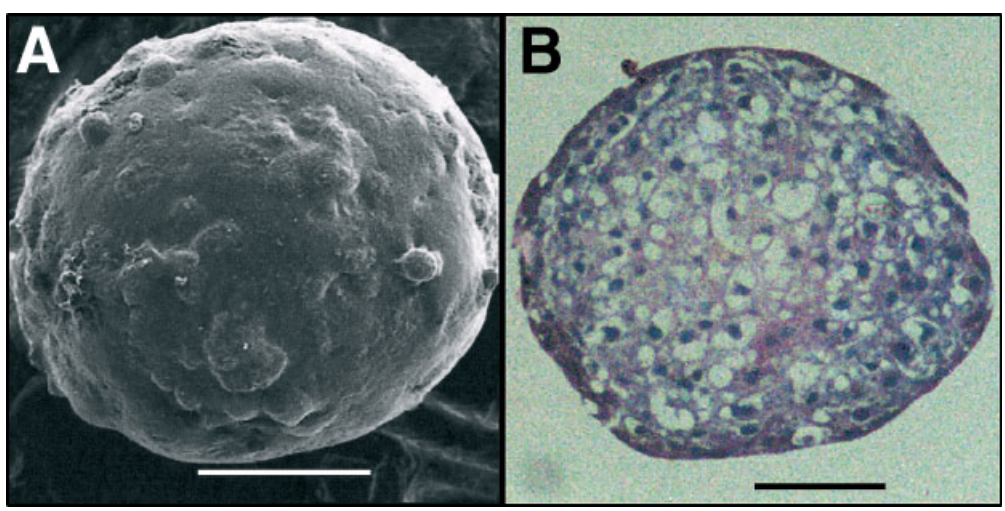

Fig. 2. SEM image and histology of liver spheroids. **A**: SEM image of a liver spheroid; the bar represents 50 μm. **B**: Histological architecture of a 6 days spheroid; the bar represents 50 μm.

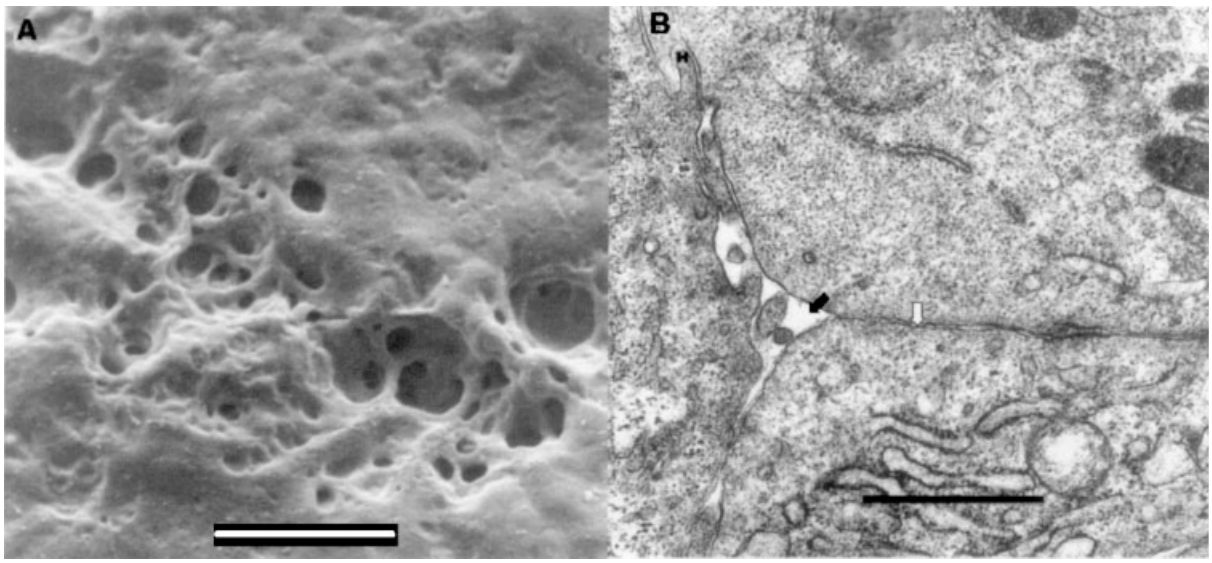

Fig. 3. Ultra-structure of liver spheroids. **A**: The boundary between cells on the surface of a mature spheroid under SEM; the bar represents 5 μm. **B**: Cell–cell boundary (white arrow) and junction (black arrow) and a hinge-like cell–cell contact (H) under transmission electron microscopy (TCM); the bar represents 1 μm.

difference between mature and immature spheroids except for the first 24 h, which was significantly lower than that at 48 h ($P < 0.01$) but not significantly different from day 6.

Galactose and pyruvate are two major energy sources and their relative consumption in molar ratio was approximately 1:2, respectively.

Glucose Secretion

The kinetic change of glucose secretion from liver spheroids during spheroid formation and maintenance is shown in Figure 4C. Glucose secretion by immature spheroids during the first 3 days was significantly higher ($P < 0.01$) than that in mature spheroids (day 6 value). It gradually declined and was maintained at a relatively stable level in mature spheroids over the 21 days period of observation.

Albumin Secretion

Albumin secretion of liver spheroids during spheroid formation and maintenance is shown in Figure 4D. The sharp fall in albumin secretion within the first 2 days was followed by a recovery phase in the mature spheroids and then gradually decreased to a lower level from day 15. This pattern was similar to that seen in the two batches of spheroids from two rats. There were differences between the two rats in the recovery phase but the change in immature spheroids was the same.

Total Protein

Each of the values of the different parameters was corrected against the total protein of spheroids in each well. The total protein change in individual wells over time in culture is shown in Figure 4E (data reflects at each time-point 18 individual wells from three different batches of liver spheroids, 6-wells/batch). A significant decrease in total protein was observed within the first 2 days after plating ($P < 0.01$) and declined gradually thereafter.

Cellular γ-GT Activity

γ-GT activity at 24 h in immature spheroids was just detectable. Its activity within the first 3 days was significantly lower ($P < 0.01$, compared with the 6 day value) than in mature spheroids (Fig. 5A). It showed a gradual increase as the spheroids formed and was maintained at a higher level in mature spheroids.

Cellular LDH Activity

LDH activity in immature spheroids was generally lower than that in mature spheroids from day 6 to 15 ($P < 0.01$, compared with the value at day 6). It showed a gradual increase as the spheroids formed and matured and was maintained at a relatively high level from day 6 to 15 (Fig. 5B). After 15 days, LDH activity significantly ($P < 0.05$ or 0.01, compared with the day 15 value) decreased to a level similar to that in immature spheroids.

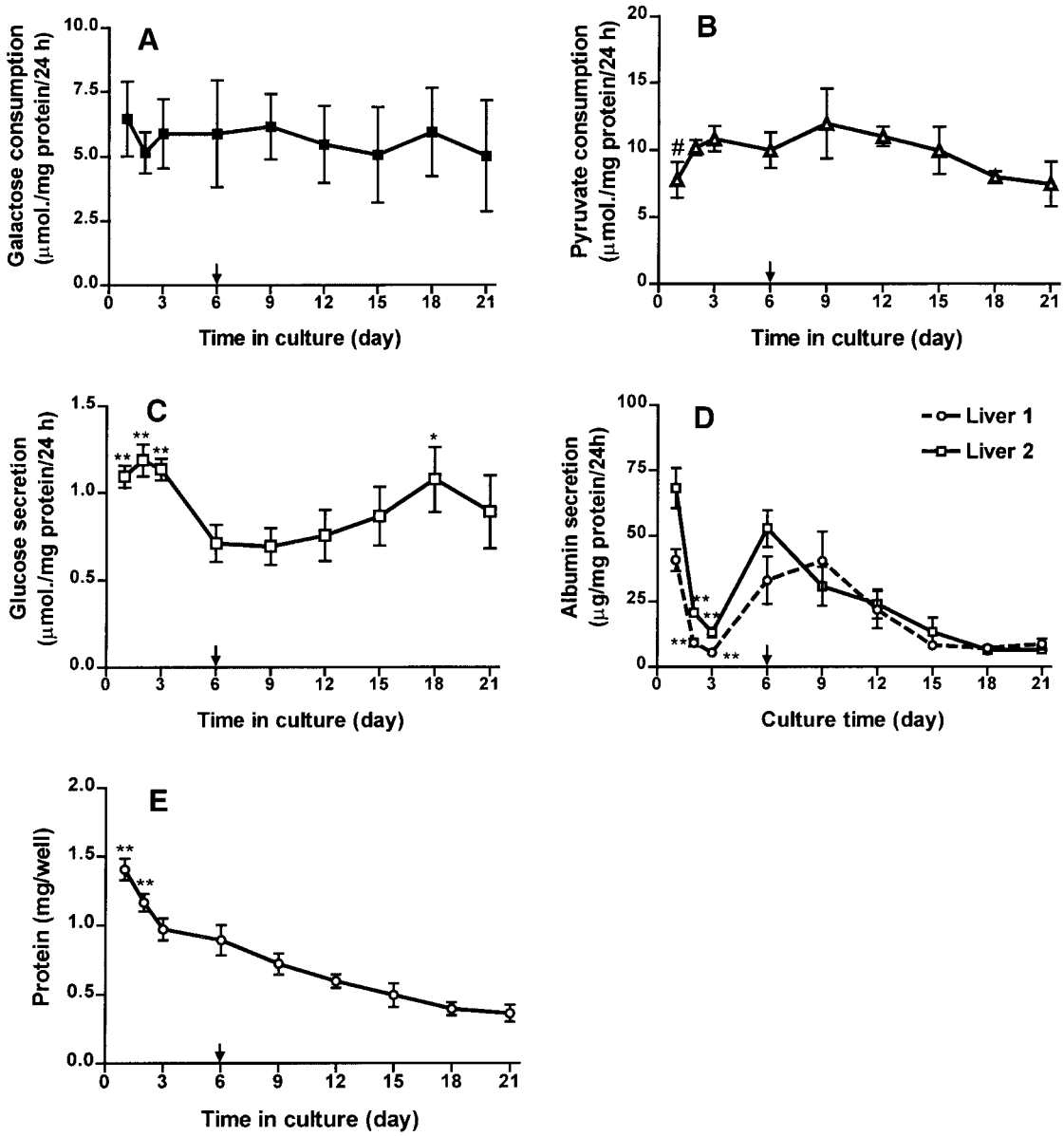

Fig. 4. Kinetic changes of galactose and pyruvate consumption, glucose and albumin secretion, and total protein during liver spheroid formation and maintenance in culture. **A**: galactose consumption; **B**: pyruvate consumption; **C**: glucose secretion; **D**: albumin secretion; **E**: protein. Values are expressed as mean ± SD, n = 6 (wells). The protein value in E at each time-point is the average of 18-wells of liver spheroids from three individual batches. *: $P < 0.05$; **: $P < 0.01$, compared with the value of 6 days spheroids. #: $P < 0.01$, compared with the value of 2 days spheroids. The arrows indicate the time-point between immature and mature periods.

Cellular GPT Activity

GPT activity in liver spheroids during spheroid formation and maintenance is shown in Figure 5C. GPT activities in immature and mature spheroids were significantly different. The highest GPT activity was detected at 24 h after cell isolation and thereafter decreased until day 6, after which GPT was maintained at a low but relatively stable level. The activity level in the first 3 days was significantly higher ($P < 0.01$) than that at day 6. The highest GPT activity in immature spheroids was three-fold higher than the average value in mature spheroids.

Cellular GOT Activity

GOT activity in immature spheroids as shown in Figure 5D was significantly higher than that at day 6. It was maintained at a relatively stable

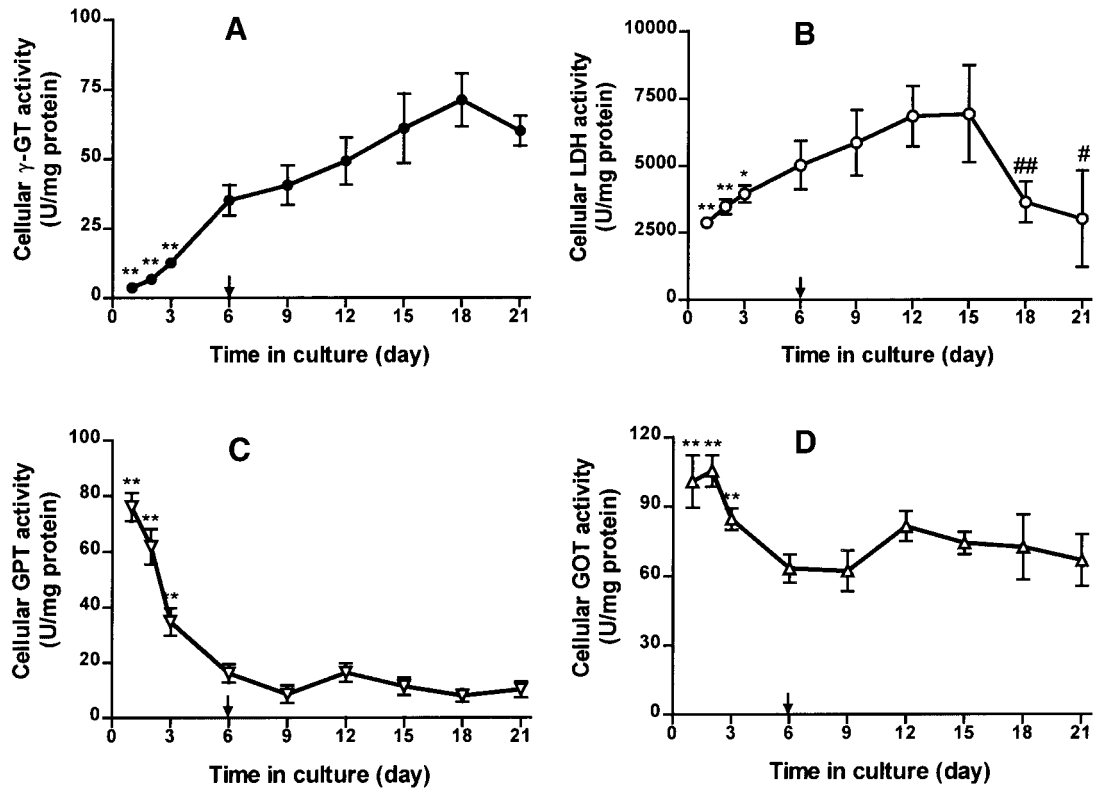

Fig. 5. Changes of some enzyme activities during liver spheroid formation and maintenance in culture. **A**: cellular γ-GT activity; **B**: cellular LDH activity; **C**: cellular GPT activity; **D**: cellular GOT activity. Values are expressed as mean ± SD, n = 6 (wells). *: $P < 0.05$; **: $P < 0.01$, compared with the value of 6 days spheroids. #: $P < 0.05$; ##: $P < 0.01$, compared with the value of 15 days spheroids. The arrows indicate the time-point between immature and mature periods.

level in mature spheroids. The highest GOT activity in immature spheroids was 50% higher than the average value in mature spheroids. On average, the cellular GOT activity was three-fold higher than GPT in mature spheroids.

DISCUSSION

It is known that liver cell isolation procedures and the subsequent culture technique significantly affects cell functionality [Lin et al., 1995; Niwa et al., 1996; Juillerat et al., 1997]. The advantages of spheroid culture over monolayer culture in maintaining cell longevity and functionality indicate that the way of cellular organization and maintenance was an important factor for functional performance of liver cells cultured in vitro [Juillerat et al., 1997]. However, little was known about the extent of biochemical and functional changes of liver cells following cell isolation and during reorganization in in vitro culture conditions and long-term maintenance. This study clearly shows that liver cells undergo a period of biochemical and functional turbulence within the first couple of days following cell isolation and then tended to maintain a relatively stable functional status in mature spheroids (>5 days) for periods upto 15–21 days. Galactose and pyruvate consumption was maintained at a relatively stable level throughout the period of observation. However, glucose secretion and cellular GPT and GOT activities were higher in immature spheroids and decreased upto day 6 and remained stable thereafter. Cellular γ-GT and LDH activities were initially undetectable or low and increased as spheroids matured and albumin secretion decreased rapidly within the first 2 days and recovered as spheroids matured.

This shows that the cell isolation and culture process initiate gross biochemical modulation in liver cells. When cells resumed cell–cell contacts and a stable histological environment was achieved in mature spheroids, most functions tended to be stabilized or recovered and were maintained for a period of time. This

biochemical and functional profile during liver spheroid formation and maintenance provides a comprehensive picture of the functional status of liver spheroids over time in culture.

Liver spheroid formation was divided into two stages in this study according to the morphological formation process: immature and mature. The immature stage (1–5 days) reflected the transitional process from single cells to morphologically stable spheroids, and in the mature stage (>5 days) the size and shape were relatively stable and regular. The concept of a 'mature' spheroid was originally described by Hansen et al. [1998] based upon morphology alone. The present study demonstrates that morphological maturity of liver spheroids is accompanied by some significant functional and biochemical changes. Thus the classification of immature and mature spheroids can provide a useful guide about the functional status of spheroids at different stages. The identification of a functionally stable period in mature spheroids is valuable in using spheroids as an in vitro model. The period from day 6 to 15 is recommended for functional and toxicological tests. This recommendation is strongly supported by the kinetic change in albumin secretion. After day 15, a 'late stage' of mature spheroids may be observed, but this does not mean that other functions of liver spheroids undergo significant changes after day 15.

The liver plays an important role in maintaining blood glucose concentration and is also the major organ responsible for converting galactose into glucose and synthesizing glucose from other substances such as pyruvic acid, amino acids [Zubay, 1988]. Galactose conversion is a liver-specific function used as a clinical test called the Galactose Tolerance Test [Varley et al., 1980]. The present study demonstrated that liver spheroids retain the functions of taking up galactose and releasing glucose into the medium. These functions were maintained across the 3-week period of observation. Glucose secretion mentioned in this study is a spontaneous function of hepatocytes because spheroids were cultured in a serum-free and glucose-free medium without neural and/or hormonal influences. It has been shown that bio-artificial liver prepared from hepatocyte spheroids can maintain normal blood glucose levels (50–105 mg/dl) in experimental animals [Mathews and van Holde, 1990]. This suggests that glucose secretion by spheroids can be modulated by hormones in vivo. It is not clear how much the glucose transformed from galactose contributed to the secreted glucose, but galactose transformation did play a part in spontaneous glucose secretion from spheroids because increasing the galactose concentration significantly increased glucose secretion [unpublished data].

Pyruvate uptake in liver spheroids has not previously been studied. Pyruvate in liver can be used for either gluconeogenesis to generate glucose or metabolized to produce ATP through the Krebs Cycle by way of acetyl-CoA in mitochondria [Zubay, 1988]. We have shown, using magic angle spinning and high resolution ^{1}H NMR spectroscopy, that glycogen was not detected in either hepatocytes or liver spheroids, whereas strong signals for glycogen were detected in intact liver tissue [Bollard et al., 2002]. This may suggest that gluconeogenesis and glycogenesis are unlikely to be the major metabolic pathway of pyruvate and pyruvate is likely to be used as an energy source. Mitochondrial disease affects pyruvate consumption [Chariot et al., 1994] and hence pyruvate consumption may indirectly reflect mitochondrial function in liver spheroids. It, therefore, has some utility as an endpoint to determine spheroid functionality.

Albumin synthesis is commonly measured in the evaluation of liver in vitro models. Culture conditions seem to be a key factor that influence the expression of the albumin gene and production of the protein in hepatocytes. It was shown that albumin synthesis decreased rapidly and was lost within 4 days in uncoated hepatocyte monolayer culture [Hou et al., 2001] and was maintained for a few weeks in spheroids or poly-N-p-vinylbenzyl-D-lactonamide-coated culture [Hou et al., 2001; Yamada et al., 2001]. Reported albumin release from hepatocytes cultured under different conditions was variable [Juillerat et al., 1997; Dilworth et al., 2000; Hou et al., 2001; Yamada et al., 2001]. The pattern of albumin secretion observed in the present study was similar to that reported by Dilworth et al. [2000] and agrees with the recovery pattern of albumin secretion by spheroids between day 2 and 7 reported by Juillerat et al. [1997].

It has been shown that NO synthesis induced by lipopolysaccharide plays an important role in inhibiting albumin gene expression [Milosevic et al., 1999]. Our data showed that NO synthesis

reached the highest level within the first 24 h after cell plating and decreased to a lower but relatively stable level thereafter as described in the companion article [Xu et al., 2003]. Significant NO synthase expression and an increase in NO synthesis were observed 4–5 h after cell isolation [Wang et al., 1998; Nicholls-Grzemski et al., 1999]. However, the present study showed that albumin secretion within the first 24 h was not as low as expected. The explanation for this appears to be that NO can inhibit albumin synthesis but does not affect its secretion and hence stored albumin in cells can be released continuously into the medium until it is depleted. The low levels of albumin secretion at 48 and 72 h are, therefore, likely to be due to both depletion of stores and a low level of synthesis. Therefore, albumin secretion may serve as a suitable parameter to reflect functional change after isolation and recovery as spheroids mature.

Total protein of spheroids in each well can indirectly reflect the total viable cell number. Total protein change during liver spheroid formation and maintenance decreased over the days of observation. This pattern of change likely reflected a gradual loss of cells injured during the enzymatic cell isolation procedure and perhaps other causes yet to be elucidated. The kinetic change of total protein shown in Figure 4E contrasts with other functional and biochemical changes measured. It suggests that the patterns of change of other parameters were unlikely to be affected by total protein losses.

This study showed that cellular LDH, γ-GT, GPT, and GOT activities in liver cells changed in various ways as spheroids formed. These four enzymes are often used as indicators of cell membrane integrity in hepatic cytotoxicity studies and hence it is important to know their profile during spheroid formation and maintenance in culture. Typically, γ-GT activity underwent a loss and recovery pattern. This suggests that when γ-GT is used as a cytotoxic marker in hepatotoxicity tests using liver spheroids, mature spheroids will be more suitable than immature ones because in immature spheroids γ-GT activity is too low to be detected with confidence.

Many factors may account for the turbulent performance of immature liver spheroids. The increase in NO synthesis after plating may play a key role in modulating the functional turbulence during spheroid formation as described in the companion article [Xu et al., 2003]. High levels of NO can trigger many functional changes [Alexander, 1998; Wang et al., 1998; Nicholls-Grzemski et al., 1999]. Further studies are needed to elucidate the mechanism(s) of functional cellular changes during liver spheroid formation.

In conclusion, liver cells undergo dramatic biochemical changes during spheroid formation. By day 5–6, mature spheroids are formed and most functions have recovered and are maintained at relatively stable levels. The stable period in terms of functionality and biochemical performance is at a minimum during days 6–15. This is essential information for the appropriate application of liver spheroids in in vitro studies.

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REFERENCES

Alexander B. 1998. The role of nitric oxide in hepatic metabolism. Nutrition 14:376–390.

Bollard M, Xu J, Purcell WM, Griffin J, Holmoes E, Quik C, Nicholson J. 2002. Metabolic profiling of liver spheroids using ^{1}H NMR spectroscopy: Effects of D-galactosamine toxicity. Chem Res Toxicol 15:1351–1359.

Cabaud PG, Wroblewski FA. 1958. Colorimetric measurement of lactic dehydrogenase activity of body fluids. Am J Clin Pathol 30:234.

Chariot P, Ratiney R, Ammi-Said M, Herigault R, Adnot S, Gherardi R. 1994. Optimal handling of blood samples for routine measurement of lactate and pyruvate. Arch Pathol Lab Med 118:695–697.

Dilworth C, Hamilton GA, George E, Timbrell JA. 2000. The use of liver spheroids as an in vitro model for studying induction of the stress response as a marker of chemical toxicity. Toxicol Vitr 14:169–176.

Friedemann T, Haugen GE. 1943. Pyruvic acid II: The determination of ketoacids in blood and urine. J Biol Chem 147:415.

Gosnell JE, Wong CB, Kumwenda ZL, Welch WJ, Harris HW. 2002. Extracellular matrix regulates the hepatocellular heat shock response. J Surgic Res 91:43–49.

Hansen LK, Hisao C-C, Friend JR, Wu FJ, Bridge GA, Remmel RP, Cerra FB, Hu WS. 1998. Enhanced morphology and function in hepatocyte spheroids: A model of tissue self-assembly. Tissue Engin 4:65–74.

Hou D-X, Arimura M, Fukuda M, Oka T, Fujii M. 2001. Expression of cell adhesion molecule and albumin gene in primary culture of rat hepatocytes. Cell Biol Internat 25:329–344.

Hu WS, Friend JF, Wu FJ, Sielaff T, Peshwa MV, Lazar A, Nyberg SL, Remmel RP, Cerra FB. 1997. Development of a bioartificial liver employing xenogeneic hepatocytes. Cytotechnol 23:29–38.

Iijima H, Nakazawa K, Mizumoto H, Matsushita T, Funatsu K. 1998. Formation of a spherical multicellular aggregate (spheroid) of animal cells in the pores of polyurethane foam as a cell culture substratum and its application to a hybrid artificial liver. J Biomater Sci Polym Ed 9:765–778.

Juillerat M, Marceau N, Coeytaux S, Sierra F, Kolodziejczyk E, Guigoz Y. 1997. Expression of organ-specific structures and functions in long-term cultures of aggregates from adult rat liver cells. Toxicol Vitr 11: 57–69.

Khalil M, Shaiat-Panahi A, Tootle R, Ryder T, McCloskey P, Roberts E, Hodgson H, Selden C. 2001. Human hepatocyte cell lines proliferating as cohesive spheroid colonies in alginate markedly upregulate both synthetic and detoxificatory liver function. J Hepatol 297:68–77.

Landry J, Bernier D, Ouellet C, Goytte R, Marceau N. 1985. Spheroidal aggregate culture of rat liver cells: Histotypic reorganization, biomatrix deposition, and maintenance of functional activities. J Cell Biol 101:914–923.

Lazar A, Mann HJ, Remmel RP, Shatford RA, Cerra FB, Hu WS. 1995a. Extended liver-specific functions of porcine hepatocyte spheroids entrapped in collagen gel. In Vitro Cell Dev Biol Anim 31:340–346.

Lazar A, Peshwa MV, Wu FJ, Chi CM, Cerra FB, Hu WS. 1995b. Formation of porcine hepatocyte spheroids for use in a bioartificial liver. Cell Transplant 4:259–268.

Lin KH, Maeda S, Saito T. 1995. Long-term maintenance of liver-specific functions in three-dimensional culture of adult rat hepatocytes with a porous gelatin sponge support. Biotechnol Appl Biochem 21:19–27.

Mathews CK, Van Holde KE. 1990. Biochemistry. Redwood City, Canada: Benjamin/Cummings Publishing Company, Inc.

Milosevic N, Schawalder H, Maier P. 1999. Kupffer cell-mediated differential down-regulation of cytochrome P450 metabolism in rat hepatocytes. Eur J Pharmacol 368:75–87.

Nakazawa K, Matsushita T, Funatsu K. 1997. Prolonged lidocaine metabolizing activity of primary hepatocytes with spheroid culture using polyurethane foam as a culture substratum. Cytotechnol 24:235–242.

Nicholls-Grzemski FA, Timenstein MA, Fariss MW. 1999. Time-dependent production of nitric oxide by rat hepatocyte suspensions. Biochem Pharmacol 57:1223–1226.

Niwa T, Koid N, Tsuji T, Imaoka S, Ishibashi F, Funae Y, Katagiri M. 1996. Cytochrome P450s of isolated rat hepatocytes in spheroids and monolayer cultures. Res Communicat Molecu Pathol Pharmacol 91:372–378.

Peshwa MV, Wu FJ, Follstad BD, Cerra FB, Hu WS. 1994. Kinetics of hepatocyte spheroid formation. Biotechnol Prog 10:460–466.

Roberts RA, Soames AR. 1993. Hepatocyte spheroids: Prolonged hepatocyte viability for in vitro modelling of non-genetic carcinogenesis. Fundam Appl Toxicol 21:149–158.

Sakai Y, Naruse K, Naagashima I, Muto T, Suzuki M. 1996. Large-scale preparation and function of porcine hepatocyte spheroids. Int J Artif Organs 19:294–301.

Sipes IG, Fisher RL, Smith PF, Stine ER, Gandolfi AJ, Brendel KA. 1987. Dynamic liver culture system—A tool for studying chemical biotransformation and toxicity. Arch Toxicol 11:20–33.

Tzanakakis ES, Hansen LK, Hu WS. 2001. The role of actin filaments and microtubles in hepatocyte spheroid self-assembly. Cell Motil Cytoskeleton 48:175–189.

Varley H, Gowenlock AH, Bell M. 1980. Practical clinical biochemistry. London: William Heinemann Medical Books Ltd.

Vonk RJ, Jekel PA, Meijer DKF, Hardonk MJ. 1978. Transport of drugs in isolated hepatocytes the influence of bile salts. Biochem Pharmacol 27:397–405.

Walker TM, Rhodes PC, Westmoreland C. 2000. The differential cytotoxicity of methotrexate in rat hepatocyte monolayer and spheroid cultures. Toxicol Vitr 14:475–485.

Wang H, Gao X, Fukumoto S, Tademoto S, Sato K, Hirai K. 1998. Post-isolation inducible nitric oxide synthase gene expression due to collagenase buffer perfusion and characterization of the gene regulation in primary cultured murine hepatocytes. J Biochem (Tokyo) 124: 892–899.

Wilson I, Gamble M. 2002. The hematoxilins and eosin. In: Bancroft JD, Gamble M, editors. Theory and practice of histological techniques. London: Churchill Livingstone. pp 125–138.

Xu J, Ma M, Purcell WM. 2002. Optimising the enzymatic determination of galactose in the culture media of rat liver and HepG2 cell spheroids. Analyt Biochem 311: 179–181.

Xu J, Ma M, Purcell WM. 2003. Biochemical and functional changes of rat liver spheroids during spheroid formation and maintenance in culture: II. Nitric oxide synthesis and relate changes. J Cell Biochem (in press).

Yagi K, Tsuda K, Serada M, Yamada C, Kondoh A, Miura Y. 1993. Rapid formation of multicellular spheroids of adult rat hepatocytes by rotation culture and their immobilization within calcium alginate. Artific Organ 17:929–934.

Yamada K, Kamihira M, Iijima S. 2001. Self-organizaton of liver constitutive cells mediated by artificial matrix and improvement of liver functions in long-term culture. Bioche Engineer J 8:135–143.

Zubay G. 1988. *Biochemistry*. New York: Collier MacMillan Publishers.