Journal of Cellular Biochemistry 90:1176–1185 (2003)

Biochemical and Functional Changes of Rat Liver Spheroids During Spheroid Formation and Maintenance in Culture: II. Nitric Oxide Synthesis and Related Changes

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Abstract Liver cells isolated from intact tissue can reaggregate to form three-dimensional, multicellular spheroids in vitro. During this process, cells undergo a histological and environmental change. How cells respond biochemically to this change has not been studied in detail previously. We have investigated some biochemical changes in rat liver cells during the formation and maintenance of spheroids. Liver cells were isolated from male Sprague rats and spheroids cultured by a gyrotatory-mediated method. Liver cells were shown to respond to the isolation procedure and the formation of spheroids triggered histological environmental changes that increased arginine uptake, nitric oxide (NO) and urea syntheses, as well as raised levels of GSH, GSSG, glutamic acid and aspartic acid secretion within the first couple of days after cell isolation. Levels were maintained at a relatively stable level in the mature spheroids (>5 days) over the 3 week period of observation. P450 1A1 activity was lost in the first 2 days and gradually recovered thereafter. This study, for the first time, shows that liver cells after isolation and during spheroid formation actively uptake arginine and increase NO and urea syntheses. A high level of NO is likely to play an important role in modulating a series of biochemical changes in liver cells. It is considered that liver cells actively respond to the 'challenge' induced by the isolation procedure and subsequent histological environmental changes, and biochemical modulation and instability result. The stable cell–cell contacts and histological environment in mature spheroids permit and support functional recovery and maintenance in vitro. This period of stability permits the use of spheroids in toxicity studies to establish acute and chronic paradigms. J. Cell. Biochem. 90: 1176–1185, 2003. © 2003 Wiley-Liss, Inc.

Key words: liver spheroid; nitric oxide; arginine uptake

Primary liver cells isolated from intact tissue have been widely used as in vitro models in research since the first hepatocyte isolation method was described some 30 years ago [Ekwall, 1980; Strain, 1994; MacGregor et al., 2001]. Primary cell culture sought to support cell functions in vitro to mimic those found in vivo [Strain, 1999]. However, it has long been known that isolated liver cells do not perform some functions well and other functions are lost. For example, isolated liver cells lose P450 activity quickly [Lazar et al., 1995; Wu et al., 1995; Hansen et al., 1998] and show unstable functionality [McGowan et al., 1981; Nicholls-Grzemski et al., 1999]. These changes indicate that the cell isolation procedure not only histologically relocates cells but also induces functional changes. Efforts have been made to improve the longevity and functionality of primary cell culture by defining the media [Guguen-Guillouzo et al., 1983; Strain, 1994], enhancing the extracellular matrix [Reid et al., 1980; Strain, 1999], co-culturing hepatocytes with other types of cells [Guguen-Guillouzo et al., 1983], developing three-dimensional cultures [Landry et al., 1984; Lazar et al., 1995; Strain, 1999] and inhibiting inducible nitric oxide (NO) synthase [Donato et al., 2001]. Although these efforts improved the survival and functionality of cultured liver cells, some

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Received 23 April 2003; Accepted 29 August 2003
DOI 10.1002/jcb.10731

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fundamental questions remain to be addressed. The trigger(s) for functional change, recovery profile and optimal conditions for stabilising phenotype after cell isolation are key areas for inquiry.

Freshly isolated liver cells can reaggregate together to form three-dimensional, tissue-like spheroids under certain culture conditions. Cells in a spheroid resume cell–cell contacts [Landry et al., 1984; Peshwa et al., 1996; Yumoto et al., 1996; Tzanakakis et al., 2001] and it has been shown that liver cells in spheroids can survive from a few weeks to several months and maintain functions much longer and better than cells cultured as monolayers [Roberts and Soames, 1993; Lazar et al., 1995; Lin et al., 1995; Wu et al., 1995; Eschbach et al., 1997; Juillerat et al., 1997; Nakazawa et al., 1997; Hansen et al., 1998]. Liver cells in spheroid culture undergo dramatic environmental changes after enzymatic isolation and resume stable cell–cell connections after spheroid formation. This feature provides a useful model to observe cellular functional changes and recovery as isolated single cells are brought together to form three-dimensional tissue-like spheroids. So far, the biochemical changes of hepatocytes during liver spheroid formation and maintenance have not been well studied. Understanding the biochemical changes of hepatocytes following histological disturbance and subsequent formation of cell–cell contacts is helpful to understand the way liver cells respond to challenges and would serve to promote the appropriate application of the liver spheroid.

This study investigated NO synthesis and related changes in liver cells during spheroid formation and maintenance to elucidate the biochemical characteristics of liver spheroids in this regard.

MATERIALS AND METHODS

Chemicals

Foetal calf serum (FCS), hepatocyte medium, penicillin, and streptomycin sulphate, standards of amino acids, *o*-phthaldialdehyde, 2,3-diaminonaphthalene (DAN), GSH and GSSG standards, metaphosphoric acid, D-L-dithiothreitol, ethoxyresorufin, resorufin, β-naphthoflavone, probenecid and other chemicals and reagents, unless otherwise indicated, were obtained from Sigma (UK).

Spheroid Culture

Livers from male Sprague rats (Charles River, UK) were perfused by a collagenase perfusion method described by Vonk et al. [1978] to release single liver cells. Cell viability was determined by trypan blue dye exclusion. Liver cell suspensions with a viability of 80% or above were used for spheroid culture. The cell suspensions were diluted with culture medium (containing Hepatocyte Medium supplemented with 5% foetal bovine serum (FBS), 200 nM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 2 mg/ml galactose) to give a cell density of 5×10^5 cell/ml. The diluted cell suspensions were plated into 6-well plates, 3 ml/well. The plates were incubated at 37°C on a gyrotatory-shaker placed within a 5% CO_2 incubator. The shaker rotation speed was initially set to 83 rpm for the first 24 h and reduced to 77 rpm thereafter.

Sample Collection and Preparation

Sample collection. Liver spheroids were maintained in culture medium and the medium totally replaced with serum-free medium (culture medium without FBS) 24 h before sample collection at each of the following time points: 1, 2, 3, 6, 9, 12, 15, 18 and 21 days and one plate (6-wells) of liver spheroids was used at each time point. Medium and spheroid samples were collected separately.

Spheroid homogenisation and sample storage. Each well of spheroids was transferred to an ependoff tube and centrifuged at 200 g for 3 min. Medium was carefully removed using a pipette and the resultant spheroids were homogenised in 1 ml buffer containing $NaH_2PO_4 \cdot 2H_2O$, 2 mM; Na_2HPO_4, 2 mM; EDTA, 0.5 mM and NaCl, 145 mM at 4°C. Medium and homogenate samples were stored at −20°C until analysis.

Kinetics of urea secretion within 72 h. One plate (6-wells) of 6 day liver spheroids was used for this study. The old medium was totally replaced with serum-free culture medium (Hepatocyte Medium supplemented with 2 mg/ml galactose, 200 nM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulphate). Medium samples were collected at 24, 48 and 72 h (which were equivalent to 7, 8 and 9 day spheroids, respectively) after changing the medium. A 100 μl aliquot of medium was taken from each well at each time point.

Spheroids were collected at the end of the experiment (72 h).

Assays

Protein assay. Total protein was determined with a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, UK, Cat. 600-0005) based on the method developed by Bradford [1976]. Protein reagent was diluted 1:5 with distilled water. A 10 μl aliquot of spheroid homogenate was added to each well of a 96-well plate (ThermoQest SEG Ltd., UK) followed by 200 μl of the diluted protein reagent. The plate was allowed to stand for 3–5 min at room temperature. The absorbance was read on a Multiscan RC microplate reader (Multiscan RC, Labsystems, Finland) at 595 nm.

Urea determination. Urea in the medium was determined using a Urea Nitrogen kit (Cat. 640-A, Sigma). The method was modified in our laboratory to be suitable for a 96-well plate format. Briefly, medium samples were diluted with PBS at medium/PBS 1:4. Either standard or sample of 10 μl was added to each well of a 96-well plate (run in duplicate) followed by 25 μl urease solution. The plate was shaken to mix the contents and allowed to stand at room temperature for 20 min. The following reagents were then added to each well in order: 50 μl phenol nitroprusside sodium, 50 μl alkaline hypochlorite solution and 200 μl distilled water. The plate was shaken to mix and kept at room temperature for 30 min. The absorbance was read on a microplate reader at 590 nm. Medium that had not been incubated with spheroids was used as a blank control.

Determination of amino acids in the medium. Amino acids were determined by a modified *o*-phthaldialdehyde (OPA) derivatization method [Antonine et al., 1999]. Briefly, each medium sample of 100 μl was mixed with 100 μl 5% TCA in an ependoff tube. The mixture was centrifuged at 10,000 g for 5 min. Supernatant of 20 μl was diluted with 780 μl 0.05 M sodium phosphate buffer, pH 6.5. Diluted sample supernatant of 100 μl was mixed with 200 μl OPA/2-mercaptoethanol derivatisation solution (containing 100 mg OPA and 0.5 ml 2-mercaptoethanol in 100 ml 0.4 M boric acid buffer, pH 10.4) 2 min before HPLC injection.

The HPLC system (Bio-Tek Instruments, UK) was composed of a System 525 pump, a HPLC 565 Autosampler, and a HPLC 582 Column Thermostat controller; column was Sphereclonb ODS (2), 4.6 × 250 mm (Phenomenomenex, UK); mobile phase A was 0.05 M sodium phosphate buffer (pH 6.5):methanol:tetrahydrofuran (THF) (84:15:1) and mobile phase B was 0.05 M sodium phosphate buffer:methanol (20:80). The gradient was started at 6% B and then increased to 15% B within 14 min. At 14 min, it was changed to 23% B. From 14 to 35 min, the gradient was linearly increased to 52% B and then directly increased to 90% B at 36 min and maintained at this gradient from 36 to 45 min. At 45 min, the gradient was changed back to 6% and maintained at this gradient for 5 min. The next sample was derivatised 2–3 min before the previous sample ended and injected automatically by the Autosampler. Fluorescence intensity was detected with a SFM 25 Fluorescence detector (Bio-Tek Instruments) at ex/em, 330/418 nm. Column temperature was controlled by a HPLC 582 Column Thermostat set at 35°C.

NO determination. Nitrite and nitrate were determined by a fluorometric method developed by Misko et al. [1993] with slight modificcations. Briefly, nitrite was determined by adding 100 μl medium sample and 200 μl of 2,3-diaminonaphthalene (DAN) reagent (0.05 mg/ml in 0.8 N HCl) to 1.9 ml deionised water. After mixing, the samples were incubated at room temperature in the dark for 10 min. The reaction was terminated by adding 100 μl of NaOH (2.8 N). The fluorescent intensity was measured with a Luminescence Spectrometer (LS50B, (LS50B, Perkin Elmer, USA) at ex/em 365/450 nm. The total nitrite (nitrite + nitrate) was determined by mixing 100 μl of medium sample with 100 μl 0.4 U/ml nitrate reductase in 20 mM Tris buffer, pH 7.6 and 100 μl 40 μM NADPH in Tris buffer. After 10 min incubation in the dark at room temperature, 1.7 ml water was added and followed by 200 μl DAN reagent. After mixing and incubating at 20°C in the dark for 10 min, the reaction was terminated by adding 100 μl of NaOH (2.8 N). Fluorescent intensity was measured as stated above.

Determination of GSH and GSSG in spheroids

Reduced GSH. Liver spheroids were homogenised in buffer and 100 μl homogenate deproteinised by adding 400 μl of 6% metaphosphoric acid and centrifugation at 10,000 g, 4°C for 7 min.

Total GSH. A 100 μl aliquot of spheroid homogenate was mixed with 100 μl of 25 mM D-L-dithiothreitol solution and 50 μl 0.1 M Tris buffer, pH 8.5. After 30 min at 4°C, the proteins were precipitated by addition of 800 μl 6% metaphosphoric acid (Sigma). All the samples were centrifuged at 10,000 g for 5 min and supernatants stored at −80°C until analysis. GSH and total GSH were determined with a HPLC method after OPA derivatisation developed by Cereser et al. [2001]. GSSG concentration was obtained by subtraction of GSH from the total GSH.

Determination of P450 1A1 activity. Ethoxyresorufin is a specific substrate for rat P450 1A1 and resorufin is one of its metabolites. Resorufin production represents P450 1A1 activity. Resorufin was determined using a fluorimetric assay developed by Burke and Richard [1974] with some modifications. Briefly, liver cells (in the first 24 h) or spheroids cultured in a 6-well format were incubated with culture medium containing 50 μM β-naphthoflavone, a P450 1A1 inducer, for 24 h. After 24 h induction, each well of spheroids was separated and incubated with 1.5 ml medium containing 20 μM ethoxyresorufin and 200 μM probenecid to prevent resorufin from being further metabolised. After 90 min incubation, spheroids were homogenised with the medium. An aliquot of 10 μl was taken for protein determination and the rest of the homogenate used for resorufin determination. The fluorescent intensity of resorufin was measured at em/ex 560/586 nm with a Fluorescence Spectrometer (LS-50B, Perkin-Elmer).

Statistics

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

RESULTS

Kinetics of Urea Secretion

Liver spheroids (6 days) were cultured in serum-free medium for 72 h (equal to day 6–9 spheroids) without changing the medium. The kinetic accumulation of urea in the medium is shown in Figure 1A. Urea accumulation in the medium within 48 h showed a good linear increase, indicating that hepatocytes in spheroids constantly secrete urea. After that, daily urea secretion decreased by around 25% compared with the first 2 days.

Urea Secretion From Liver Spheroids During Formation and Maintenance

The change in urea secretion during liver spheroid formation and maintenance is shown in Figure 1B. Urea secretion within the first 24 h was significantly higher than that at other periods and gradually decreased by day 6 where it reached a relatively stable level. Urea synthesis in the first 24 h was 4–5 fold higher than that at the average stable level.

NO Synthesis in Liver Spheroids

NO synthesis, indicated by nitrite and nitrate formation, in liver spheroids is shown in Figure 2. Similar to urea secretion, NO release within the first 24 h was at its highest and thereafter decreased. At day 6 and onwards, NO synthesis was decreased to a lower but

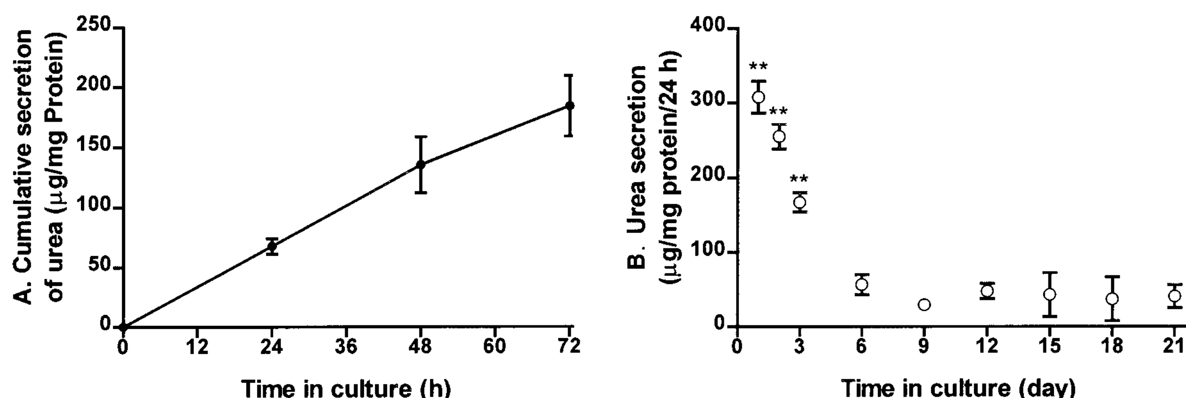

Fig. 1. Kinetics of urea secretion from mature liver spheroids over 72 h incubation and long-term maintenance. **A:** Cumulative secretion of urea within 72 h; **B:** Urea secretion during spheroid formation and maintenance. Data are the mean ± SD, n = 6. $^{**}P < 0.01$, compared with the value from day 6 spheroids.

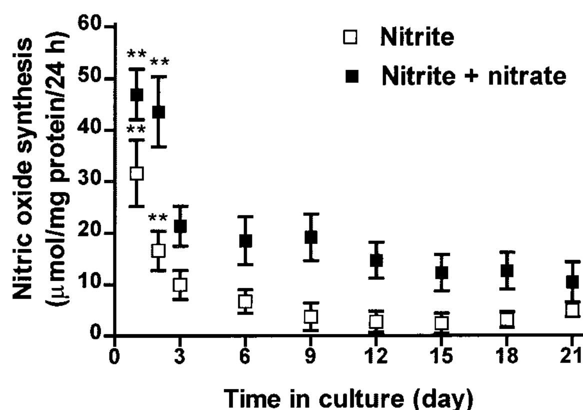

Fig. 2. Nitric oxide (NO) synthesis in rat liver spheroids during spheroid formation and maintenance. Data are the mean ± SD, n = 6. $^{**}P < 0.01$, compared with the values from day 6 spheroids.

relatively stable level, which ranged between 10 and 25% of the 24 h value. The ratio of nitrite/nitrate at 24 h was 2.1:1, whereas at day 12 it was 0.23:1. The ratio at other time points ranged between 0.23:1 and 0.88:1. Nitrite was a major form of the NO product within the first 24 h in culture as shown by the nitrite/nitrate ratios.

Amino Acid Metabolism

Seventeen amino acids were determined in the medium of liver spheroids using a HPLC method (Fig. 3). Two types of amino acid metabolism were observed in liver spheroids: secretion and consumption. Aspartic acid (peak 1) and glutamic acid (peak 2), which were not present in the control culture medium, were detected after incubation with liver spheroids. This indicates that liver spheroids can synthesis and secrete these two amino acids. Figure 4A shows their secretion in the first 24 h was highest, decreasing daily in immature spheroids, and achieving a relatively stable level in mature spheroids. The secretion of aspartic acid and glutamic acid were in parallel although glutamic acid secretion was much higher than that of aspartic acid ($P < 0.01$, compared on a daily basis). The average ratio of glutamic acid:aspartic acid was 8.8 ± 4.5:1. After day 15, aspartic acid secretion began to rise.

Among the consumed amino acids, arginine (peak 9) and alanine (peak 10) were the two most consumed amino acids as shown in Figure 4B. Arginine was the only amino acid observed that was specifically consumed by immature spheroids. It was depleted from the culture medium within the first 2 days, see Figures 3 and 4B. The plateau of arginine consumption before 48 h (Fig. 4B) indicates that all the available arginine in the medium was taken up by the spheroids. Its consumption was maintained at a relatively stable level in mature spheroids during the 21 day period of observation. The pattern of alanine consumption (Fig. 4B) represented the pattern of consumption for the other amino acids. In mature spheroids, the consumption levels of arginine and alanine were similar (Fig. 4B). The consumption of most other amino acids assayed was much lower than arginine and alanine at all time points and was relatively stable during the 21 day period of observation (data not shown).

Changes in GSH and GSSG Levels in Liver Spheroids

Figure 5 shows the kinetic changes of GSH and GSSG during spheroid formation and maintenance in culture. The levels of GSH and GSSG in liver spheroids were significantly higher within the first 3 days and decreased up to day 6, thereafter being maintained at a relatively stable level. They shared a similar pattern to NO synthesis and urea secretion. The ratio of GSH/GSSG was in the range 4.5–6.6:1 up to day 15 and thereafter was 9–11:1.

P450 1A1 Activity

The change in P450 1A1 activity over time is shown in Figure 6. It may be divided into three phases. For the first 48 h, P450 1A1 in liver spheroids did not respond to the inducer, β-naphthoflavone and its activity was very low or undetectable. From days 3 to 12, P450 1A1 was inducible and maintained at a relatively stable level of activity. After this time, P450 1A1 activity further increased. During the 21 day period of observation, P450 1A1 underwent a loss and gradually recovered.

DISCUSSION

The present study demonstrates that a series of biochemical changes in liver cells occurred after cell isolation and during spheroid formation. These significant changes indicate that cells actively respond to histological disturbance and functional and biochemical changes occur. When cells resume cell–cell contacts in mature spheroids (>5 days), those functions measured had recovered and were maintained

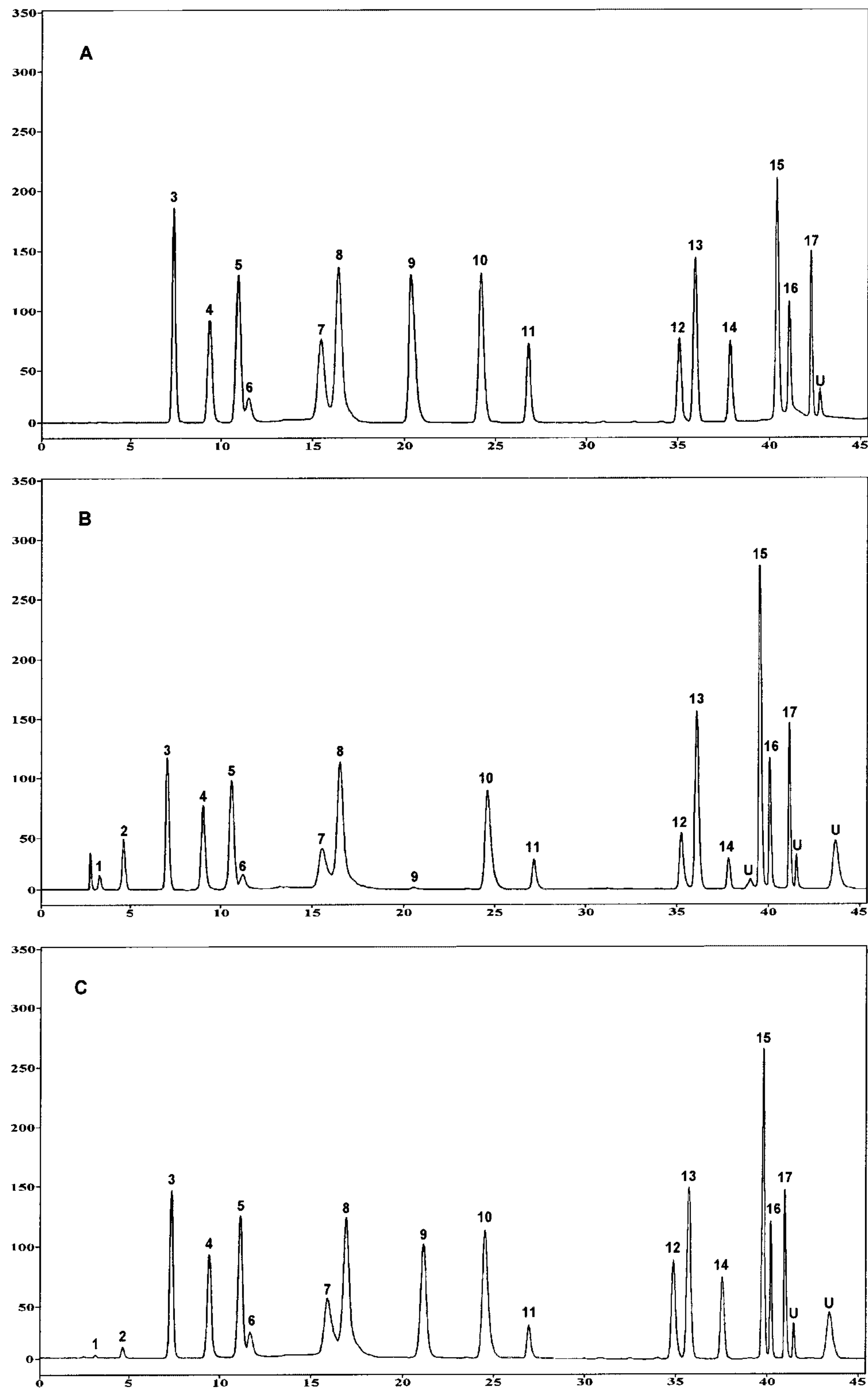

Fig. 3. HPLC graphs of amino acid analysis. **A**: Control medium without co-culture with liver spheroids. **B**: Medium co-cultured with 1 day spheroids for 24 h. **C**: Medium co-cultured with 6 day spheroids for 24 h. Peaks (1) L-aspartic acid, (2) glutamic acid, (3) L-arsparagine, (4) L-serine, (5) L-histidine, (6) L-glutamine, (7) L-threonine, (8) glycine, (9) L-arginine, (10) L-alanine, (11) L-tyrosine, (12) L-methionine, (13) L-valine, (14) L-phenylalanine, (15) L-isoleucine, (16) L-leucine, and (17) L-lysine. U, unidentified peak.

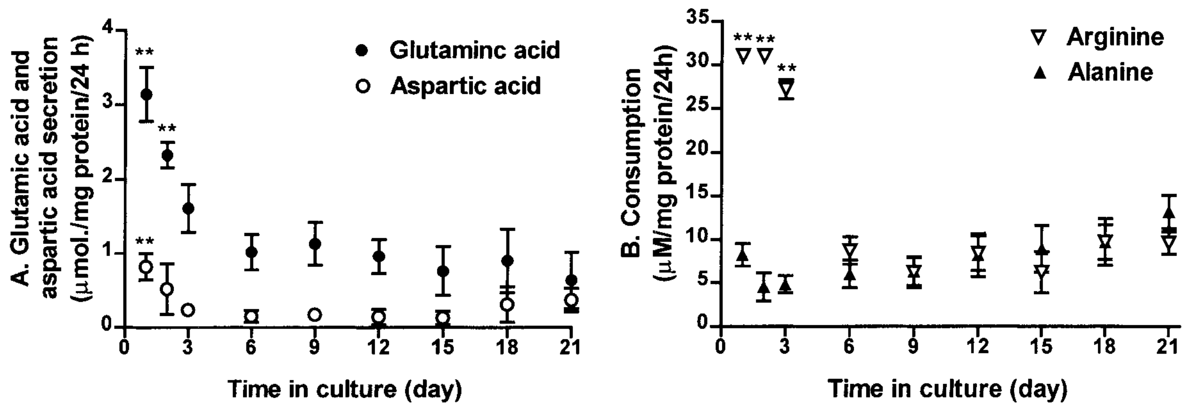

Fig. 4. Amino acid metabolism in liver spheroids during spheroid formation and maintenance. **A**: Glutamic acid and aspartic acid secretion from rat liver spheroids during spheroid formation and maintenance. **B**: Arginine and alanine consumption during spheroid formation and maintenance. Arginine was depleted within the first 2 days during liver spheroid formation. Data are the mean ± SD, n = 6. $^{**}P < 0.01$, compared with the values from day 6 spheroids.

as indicated by P450 1A1 and other parameters such as albumin and γ-GT as described in the companion manuscript [Ma et al., 2003]. This change indicates that a stable histological environment is an important factor for functional recovery and maintenance of hepatocytes in vitro. Although time factors may also contribute to the functional differences between immature and mature spheroids, hepatocytes cultured in monolayers without medium or matrix modification tend to lose some functions during long-term maintenance [Reid et al., 1980; Hou et al., 2001; Yamada et al., 2001].

NO in hepatocytes is synthesised by an inducible NO synthase [iNOS, Alexander, 1998]. It is an intermediate of arginine oxidation in cells and nitrite and nitrate are its two major products. It was reported that the ratio of nitrite/nitrate is variable from 3:2 to 1:2.2 dependent upon the concentration of •N=O [Stuehr and Marletta, 1987; Marletta et al., 1998]. The present study showed that the ratio of nitrite/nitrate was changeable during spheroid formation. More nitrite was secreted than nitrate within the first 24 h and the ratio of nitrite/nitrate was gradually reduced as the total NO concentration decreased. This suggests that a high concentration of NO tends to form more nitrites than nitrates in liver spheroids and the change in nitrite levels may be more sensitive than nitrate to reflect NOS induction in liver cells.

It has been shown that histological disturbances can trigger significant NO synthesis.

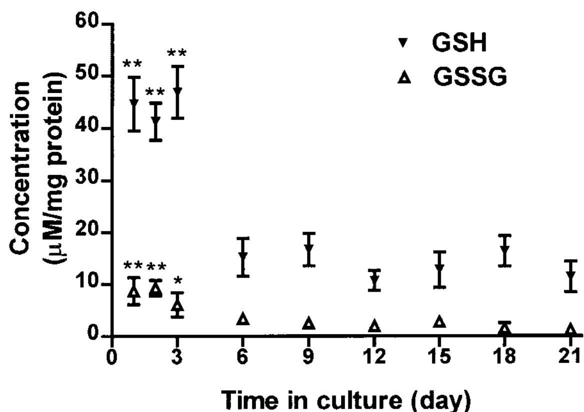

Fig. 5. GSH and GSSG levels in rat liver spheroids during spheroid formation and maintenance. Data are the mean ± SD, n = 6. $^{*}P < 0.05$; $^{**}P < 0.01$, compared with the values from day 6 spheroids.

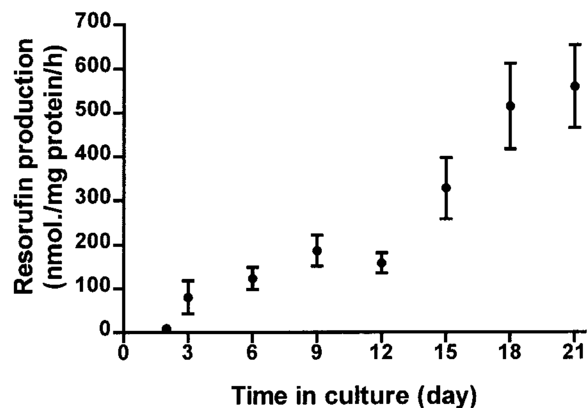

Fig. 6. Changes in P450 1A1 activity in rat liver spheroids during spheroid formation and maintenance. Data are the mean ± SD, n = 6.

The increase in NO synthesis was observed 5 h after hepatectomy [Carnovale et al., 2000]. Inducible NOS mRNA expression and NO synthesis in hepatocytes were increased 4 h after collagenase isolation [Wang et al., 1998; Nicholls-Grzemski et al., 1999]. In this study, most cells remained single or formed very small aggregates of a few cells 4 h after isolation. This mirrors the initial cell response induced in cells by isolation when cultured either as monolayers [Wang et al., 1998] or spheroids. The present study further demonstrated a high level of NO synthesis coupled with high levels of arginine uptake and urea synthesis took place at the initial stage of liver spheroid culture. NO and urea syntheses and arginine metabolism are interrelated since arginine is the common substrate for both urea and NO syntheses. The relative production of urea and NO depends upon many factors and is involved in a complex regulation process. It was shown in hepatocytes that when NOS was induced, more arginine was used for NO synthesis and urea synthesis was inhibited by 45%, but the inhibition of urea production was overcome by a high concentration of arginine [Stadler et al., 1995]. This was due to the fact that when a high level of surplus arginine is available inside hepatocytes, urea synthesis will be promoted. Although arginine can be synthesised through the ornithine cycle in hepatocytes, taking up arginine from the medium will be a quicker and more economical way than synthesis. This may explain why arginine was depleted from the medium in the first couple of days after plating liver cells for spheroid culture.

The interrelated changes of arginine uptake and NO and urea syntheses may induce subsequent changes. NO can activate soluble guanylate cyclase to raise cyclic guanosine monophosphate (cGMP) levels. This signal transduction can elicit many actions [Alexander, 1998]. The loss of P450 1A1 observed in the present study and inhibition of albumin synthesis as described in the companion manuscript [Ma et al., 2003] and reported by others [Khatsenko et al., 1998; López-García, 1998; Milosevic et al., 1999] are an indication of the effects of NO. Thus, the increase of NO synthesis is a reminder of the cell reaction and subsequent functional and biochemical changes after enzymatic isolation. Interestingly, we showed that the affected functions in liver spheroids recovered as NO synthesis decreased, which has not been previously reported. Efforts have been made to maintain hepatocyte functions after isolation by inhibiting NOS [López-García, 1998; Donato et al., 2001]. We showed the natural kinetic changes of NO synthesis and recovery of liver functions during liver spheroid formation and maintenance and their important implication for appropriate use of the liver spheroid model.

Amino acid metabolism may also be affected as indicated by glutamic acid and aspartic acid secretion observed in this study. Glutamic acid can combine with NH_3 to form glutamine in other tissues and glutamine then carries the NH_3 to the liver for urea or other non-essential amino acid syntheses. Glutamine has been termed an ammonium carrier [Zubay, 1988]. Asparagine may play a similar role, but to a lesser extent than glutamine. Alanine was another of the most consumed amino acids in liver spheroids and is also an important NH_3 carrier, mainly from the muscles [Zubay, 1988]. The uptake level of alanine in both immature and mature liver spheroids was significantly higher than most of the other amino acids but was unlikely to play a significant part in modulating cell functions during spheroid formation.

GSH plays an important role in cellular homeostasis and protects cells from free radical- and peroxidation-induced damage. This study shows that GSH levels paralleled NO synthesis. NO can react with reactive oxygen species to form highly reactive nitrogenated superoxides, ONOOH/$ONOO^{2-}$ [Davies et al., 1995; Alexander, 1998]. It has been reported that hepatectomy induced a high level of NO synthesis that was accompanied by increased lipid peroxidation [Carnovale et al., 2000]. Thus, high level NO synthesis increases the potential of peroxidation-induced damage. The present study showed that the increase in GSH and GSSG were parallel and their ratios were similar in both immature and mature spheroids. Whatever the ratio, the increase in GSSG levels may indicate oxidative stress in cells. However, the high level of available GSH in cells plays an important role in preventing cells from damage induced by excess NO synthesis. It is not clear what induced GSH synthesis in the spheroid cultures, but the parallel increases of NO and GSH and the nature of NO as a multifunctional modulator may suggest that NO plays a role in stimulating GSH synthesis. Thus, it appears

that liver cells undergo a series of biochemical reactions after isolation and during spheroid formation.

It should be pointed out that most parameters observed during spheroid formation and maintenance in this study either shown a decrease or an increase but this does not simplistically reflect good or bad functionality since the cells undergo extensive functional and biochemical changes during the formation of spheroids. It is important for an in vitro model that physiological functionality is retained over a defined period in culture. To this end, mature spheroids are ideal for use in toxicology and functional tests relevant to chronic toxicity paradigms in addition to acute toxicity tests. Understanding the characteristics of functional drift and recovery during liver spheroid formation and maintenance is important in using this in vitro model appropriately.

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