Spheroid formation and functional restoration of human fetal hepatocytes on poly-L-amino acid-coated dishes after serial proliferation

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Abstract

Primary human fetal hepatocytes proliferated in monolayer culture up to the 9th passage. During proliferation, the cells changed their morphology from a fibroblast-like shape after inoculation to an epithelia-like polygonal shape after they reached confluence. The proliferation was associated with the loss of ammonia detoxification capacity, which is essential for the function of bioartificial liver. The cells formed spheroids on a poly-L-glutamic acid- or poly-L-aspartic acid-coated polystyrene dish that had a negatively charged surface at neutral pH. However, the cells did not form spheroids on a poly-L-lysine- or poly-L-arginine-coated dish that had a positively charged surface, which is reportedly suitable to form spheroids for adult hepatocytes. The activity of cytochrome P450 (CYP 1A1, CYP1A2) of the cells in spheroid culture was about twice as high as that of the cells in monolayer culture. The ammonia detoxification activity of the cells was restored in spheroid culture by treatment with 2% dimethylsulfoxide. These results suggest that the conditions for human fetal hepatocytes to form spheroids are different from that for adult hepatocytes, and the use of poly-L-glutamic acid or poly-L-aspartic acid coating may improve spheroid culture of proliferative human fetal hepatocytes.

Abbreviations: BAL – bioartificial liver; CLMS – confocal laser microscopy; CYP – cytochrome P450; DMSO – dimethylsulfoxide; EROD – ethoxyresorufin-*O*-dealkylation; HDM – hormone-defined medium; MROD – methoxyresorufin-*O*-dealkylation; PAS – periodic acid-Schiff; PMT – photomultiplier tubes

Introduction

Until now, various bioartificial liver (BAL) systems that use primary porcine hepatocytes have been submitted for clinical trials as a 'bridge' for liver transplantations in patients with fulminant hepatic failure (Gerlach 1996; Watanabe et al. 1997). But zoonotic issues have attracted greater attention, because of the recent discovery that the pig genome

has retroviruses (Le Tissier et al. 1997) that can infect human cells *in vitro* (Patience et al. 1999).

Although primary human hepatocytes are an ideal source for BAL, the shortage of human livers available for hepatocyte isolation limits this method, because the BAL system needs many hepatocytes of more than 1×10^{10} cells (Khalil et al. 2001), and primary human hepatocytes from adult liver can not proliferate *in vitro*. However,

human hepatocyte cell lines established from hepatoma can replicate well *in vitro*, but these cells lose many biochemical functions of hepatocytes, such as ammonia detoxification activity and drug metabolism activity that are considered essential for BAL (Enosawa et al. 1996). A controversial subject is the possible tumorigenicity of these cells. Although the use of human fetal hepatocytes is a bioethical issue, they are a candidate as a cell source for BAL, because they include hepatoblasts as a kind of hepatic stem cell, and they may proliferate *in vitro* and keep the differentiated functions. But reports of the maintenance of the differentiated functions of the cells after proliferation are few (Yoon et al. 1999).

Spheroid culture is effective in the upregulation of liver-specific functions of primary adult hepatocytes and human hepatoma cell lines (Matsushita et al. 1991; Yamashita et al. 2001), and in the reorganization of new-born rat hepatocytes (Landry et al. 1985). This study investigated the proliferation of normal human fetal hepatocytes *in vitro* and the restoration of their functions by forming spheroids cultured on a suitable surface of a culture dish modified with poly-L-amino acids.

Materials and methods

Human fetal hepatocytes and growth medium

Human fetal hepatocytes were obtained from Dainippon Pharmaceutical Co. (Osaka, Japan) with a certification of informed consent for research use. They were originally prepared from six normal fetal livers (almost 13 weeks old) by Applied Cell Biology Research Institute (Kirkland, WA, USA). We trypsinized the cells cultured in a T-125 flask (1st passage) with 0.05% trypsin and 0.53 mM ethylenediaminetetraacetate solution for 60 s, and inoculated the cells (2nd passage) at 1.5×10^5 viable cells in CS-C medium (Cell Systems, Kirkland, WA, USA) that contained acidic fibroblast growth factor and 10% fetal bovine serum in each 35 mm culture dish (Falcon #353001, Becton Dickinson and Company, Franklin Lakes, NJ, USA). After the cells were confluent, they were trypsinized again and were stored in liquid nitrogen with cryopreservation medium containing dimethylsulfoxide (DMSO).

The cryopreserved cells were thawed at 37 °C, and were inoculated at 1.5×10^5 cells in each 35 mm culture dish. The cells were cultured until they were a confluent monolayer (3rd passage). Trypsinized cells of the 3rd passage were used in all experiments. The viable cell number was measured by using the trypan blue dye exclusion method with a haemocytometer.

Preparation of poly-L-amino acid solution and coating on the surface of a polystyrene dish

Six poly-L-amino acids were used to coat the surface of polystyrene dishes (Iwaki non-treated dish, diameter 35 mm) to investigate the optimum surface characteristics for spheroid formation of human fetal hepatocytes. Poly-L-lysine, poly-L-arginine, poly-L-glutamic acid, and poly-L-aspartic acid were dissolved in PBS at 1 mg ml⁻¹, and 1 ml of each solution was added to the dishes and were incubated at 37 °C for 1 h before use. Poly-L-leucine and poly-L-isoleucine were dissolved in 98% trifluoroacetic acid at 1 mg ml⁻¹, and 1 ml of each solution was added to the dishes as described for the other four poly-L-amino acids. The dishes coated with poly-L-leucine and poly-L-isoleucine were washed with hormone-defined medium (HDM) three times. HDM comprised Williams' medium E (Gibco, Grand Island, NY, USA) supplemented with 10 µg ml⁻¹ insulin (Sigma, St. Louis, MO, USA), 50 ng ml⁻¹ epidermal growth factor (Takara, Kyoto), 0.1 µM copper (CuSO₄·5H₂O), 3 nM selenium (H₂SeO₃), 50 pM zinc (ZnSO₄·7H₂O), 50 ng ml⁻¹ linoleic acid (Sigma), 63 µg ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin, and has been used for spheroid formation of primary hepatocytes (Koide et al. 1990). Before use, all dishes were equilibrated with HDM for 10 min at room temperature.

Spheroid formation of human fetal hepatocytes

Trypsinized human fetal hepatocytes were inoculated at a density of 6×10^5 cells in 2.0 ml of HDM in each 35 mm dish. After cultivation for 4 h at 37 °C under a humidified atmosphere of 5% CO₂ and 95% air, the medium was changed and thereafter was renewed at intervals of 2 days. The morphology of hepatocytes in spheroids was observed by using a phase-contrast microscope (Nikon Eclipse

TE300 inverted microscope), and twenty microphotographs were randomly taken on each surface of the dish coated by each of the six poly-L-amino acids at 72 h of culture. The average number of spheroids and hemispheroids in each square millimeter (spheroid density) was estimated by counting their numbers on the microphotographs. Morphological judgment of spheroids and hemispheroids was according to Koide et al. (1990).

In situ confocal microscopic measurement of cytochrome P450 activity

Cytochrome P450 (CYP) activities of the cells were specifically induced by 25 μ M β -naphthoflavone in HDM for 48 h culture (Okey et al. 1986) and were measured *in situ* as ethoxyresorufin-*O*-dealkylation (EROD) activity and methoxyresorufin-*O*-dealkylation (MROD) activity by using confocal laser microscopy (CLMS) (Leica TCS-SP, Heidelberg, Germany) with a 10 $\times$ /0.45 or 20 $\times$ /0.75 objective lens. The CLMS was equipped with two photomultiplier tubes (PMT) for dual detection of fluorescent images and one transmission detector. TCS NT software (Leica) running on a Windows NT workstation was used to receive and store the data. A 543 nm light beam from a He-Ne laser was used to excite the samples to measure EROD and MROD activities. Emitted fluorescence was detected at wavelengths from 555 to 660 nm by using a multi-band detector system. The sensitivity of the PMT was adjusted to avoid intensity saturation and was held constant throughout all scans.

Before observation by CLMS, the culture medium was replaced with 2 ml of prewarmed assay medium consisting of HDM supplemented with 25 μ M dicumarol and 2 mM probenecid (Tzanakakis et al. 2001). Dicumarol, an inhibitor of DT-diaphorase (Lubet et al. 1985), was added to retard further metabolism of resorufin into non-fluorescent compounds. Probenecid, an ion transport inhibitor that also inhibits glucuronidation (Cretton and Sommadossi 1991), was included in the assay medium to prevent resorufin diffusion from the cells. Stock solutions of 10 mM dicumarol and 0.2 M probenecid (Sigma) were prepared in 0.5 N NaOH. The assay medium was adjusted to pH 7.2 with 0.5 N HCl solution before its use. To detect the activities, the incubation medium in 35-mm dishes was replaced with 2 ml of assay medium that

Figure 1. Serial passages of human fetal hepatocytes in CS-C medium with 10% FBS.

contained ethoxyresorufin (Molecular Probes, Eugene, OR) for EROD activity and methoxyresorufin for MROD activity. These alkoxyresorufins were prepared as a 1 mM stock solution in DMSO. The final alkoxyresorufin and DMSO concentrations in the dish were 20 μ M and 2.0% (v/v), respectively. The cells were then incubated for 5 min at 37 °C in a humidified 5% CO₂ incubator. Then the fluorescence and transmitted light images were acquired at room temperature.

The EROD and MROD activities were measured semi-quantitatively by using TCS NT software (Leica). A number of different fields was scanned and analyzed to obtain statistically significant results. After some preliminary scans, only focal planes with highest fluorescence intensities were chosen for analysis from each field. These planes were selected because they gave representative levels of EROD and MROD activities (Tzanakakis et al. 2001). By using TCS-NT, areas of spheroids or cell monolayers within each of the chosen focal planes were selected and the average fluorescence intensity was calculated. During area selection, regions having no cells were excluded. However, images from such regions were used to calculate the mean background intensity, which was subtracted from the average intensity of cell monolayers or spheroids.

Ammonia metabolizing activity measurement and other analytical methods

When we evaluated the ammonia metabolizing activity, the 4th passaged cells were inoculated in CS-C medium in a polystyrene tissue culture dish

Figure 2. Morphology of human fetal hepatocytes in a confluent monolayer.

Figure 3. Morphological changes in human fetal hepatocytes during proliferation at the 4th passage observed by phase contrast microscope.

(Falcon #353001) to form a confluent monolayer, and were also inoculated in HDM in a poly-L-glutamic acid-coated dish to form spheroids. After 4 h, each medium was changed to a fresh medium that included 2% DMSO and was cultured for 2~3 days, which induces differentiated functions of hepatocytes (Kost and Michalopoulos 1991). Then the culture medium was changed

again to a fresh medium supplemented with 1 mM NH_4Cl . The ammonia concentration was measured at 1, 2 and 3 h after incubation at 37 °C by using a commercial kit (Wako Pure Chemical, Osaka, Japan). To stain with periodic acid-Schiff (PAS), the cells were fixed with 10% formalin neutral buffer solution (Wako Pure Chemical Industries, Osaka, Japan).

Figure 4. PAS-stained images of human fetal hepatocytes during proliferation at the 4th passage.

Results and discussion

Serial proliferation of human fetal hepatocytes

The cryopreserved human fetal hepatocytes (2nd passage) were inoculated at about 1×10^5 cells in CS-C medium in each 35 mm dish and were cultured. The cells proliferated and reached confluence as a monolayer within 5 days (Figure 1, 3rd passage). Then, we tried to trypsinize and re-inoculate the cells serially until the 9th passage under the same culture conditions. The averaged maximum cell density and doubling time during these passages were about 2.0×10^6 cells in each 35 mm dish and about 20 h, respectively. Recently, proliferative hepatocytes from a newborn calf or adult rat liver have been studied (Mitaka et al. 1995; Karikusa and Sawasaki 1996; Tateno et al. 2000), but the doubling time (about 20 h) obtained from human fetal hepatocytes of this study was much faster than that of the calf and rat liver hepatocytes.

Figure 2 shows the morphology of human fetal hepatocytes in a confluent monolayer at the 4th, 7th and 8th passages. Generally, adult or mature parenchymal hepatocytes have a polygonal morphology, prominent nuclei and clear borders to the cells (Mitaka et al. 1995). At the 4th passage, many typical polygonal hepatocytes with prominent nuclei were observed. Although the number of these cells decreased gradually with serial

passages until the 9th passage, they were still observed at the 8th passage (Figure 2C).

Figure 3 shows the morphology of human fetal hepatocytes during cell growth at the 4th passage. The *in vitro* proliferation of human fetal hepatocytes was accompanied by changes in morphology. Inoculated cells had long projections similar to fibroblasts at day 1 (Figure 3A). The cells reached confluence at day 4 (Figure 3B); they were polygonal, but without prominent nuclei. After confluence, the cells were smaller compared with cells at day 4 due to intensive cell–cell interaction at day 9 (Figure 3C), and we observed typical morphology of hepatocytes with prominent nuclei and clear borders of the cells (Figure 3C).

These morphological changes during proliferation paralleled the changes in the PAS staining image of the cells (Figure 4). The inoculated cells at day 1 (Figure 4A) and the cells that were confluent at day 4 (Figure 4B) were negative for PAS staining, but after confluence at day 14 (Figure 4C), almost all cells were intensively positive for PAS staining. These results suggested that the cell morphology changed in the same way as parenchymal hepatocytes after reaching confluence, and that the glycogen synthesis of the cells also started together with the changing morphology. However, other functions, such as ammonia detoxification activity, were lost or decreased during proliferation in monolayer culture.

Figure 5. Morphology of human fetal hepatocytes cultured on the surfaces of polystyrene dishes coated with six poly-L-amino acids. (A) Poly-L-lysine; (B) Poly-L-arginine; (C) Poly-L-glutamic acid; (D) Poly-L-aspartic acid; (E) Poly-L-isoleucine; (F) Poly-L-leucine. Arrows indicate spheroids.

Spheroid formation of human fetal hepatocytes

Functions of adult primary hepatocytes rapidly decrease in monolayer culture within a week, but can be maintained more than several weeks by spheroid formation (Koide et al. 1990; Ijima et al.

1998). We preliminarily tried to form spheroids of human fetal hepatocytes after proliferation in monolayer culture by using a Primaria dish that had a positively charged culture surface and was effective for spheroid formation of adult primary hepatocytes. But spheroids of human

Figure 6. Average density of spheroids in each square millimeter formed on the dishes coated with six poly-L-amino acids. Values shown are averages $\pm$ S.D.

fetal hepatocytes did not form on the Primaria dish. Then, we examined the effect of surface characteristics of a culture dish on spheroid formation by using six poly-L-amino acids to modify the surface of the dish.

Figure 5 shows the morphologies of human fetal hepatocytes cultured in HDM on a polystyrene dish coated with the six poly-L-amino acids. On the surface coated with positively charged poly-L-lysine and poly-L-arginine at neutral medium pH, the hepatocytes formed no spheroids, similar to hepatocytes on the Primaria dish (Figure 5A, B). However, the inoculated hepatocytes migrated well on the surface coated with negatively charged poly-L-glutamic acid or poly-L-aspartic acid, that were observed by using a time-lapse video recording system (data not shown), and formed spheroids frequently (Figure 5C, D). Spheroids or hemispheroids were partially observed on the surface coated with hydrophobic poly-L-leucine and poly-L-isoleucine (Figure 5E, F).

In this study, to investigate the optimum surface condition for spheroid formation, the average density of spheroids and hemispheroids in a square millimeter was estimated from microphotographs randomly taken on each surface coated with six poly-L-amino acids (Figure 6). Densities of more than $2.0 \text{ spheroid number mm}^{-2}$ were obtained on the surface coated with poly-L-glutamic acid or poly-L-aspartic acid, which were almost twice as high as those on the surfaces coated with poly-L-leucine and poly-L-isoleucine. The densities on the surface coated with poly-L-lysine or poly-L-arginine were negligible. Consequently, the surfaces of dishes coated with poly-L-glutamic acid or poly-L-aspartic

acid were the most effective for spheroid formation of human fetal hepatocytes among the surfaces coated with six poly-L-amino acids.

Comparison of hepatic functions between monolayer and spheroids of human fetal hepatocytes

Finally, we compared hepatic functions between spheroids formed on a dish coated with poly-L-glutamic acid and a monolayer on the usual culture dish after proliferation in CS-C medium.

The level of EROD activity (CYP1A1) in cultured human fetal hepatocytes at the 4th and 5th passages was assessed *in situ* by using CLMS. Ethoxyresorufin (ER) was added to the cells, and CYP1A1 enzymes reacted with ER to produce fluorescent resorufin. Approximately 5 min after adding ER, images were collected for each sample. Figure 7 shows images of spheroids formed on the surface coated with poly-L-glutamic acid and of a monolayer on an uncoated dish. The images ($n > 10$) were analyzed by using a computer and the average fluorescence intensity of an area was calculated as a measure of the EROD activity (Figure 8). We also measured the MROD (CYP1A2) activity of spheroids and monolayer (Figure 8). In these CYP activities, spheroids showed more than twofold higher activities compared with monolayer culture.

Figure 9 shows the ammonia detoxification activity of cells in a monolayer and in spheroid cultures. DMSO promotes the differentiation of embryonic rat liver cells (Germain et al. 1988). First, we measured the activities in a monolayer and in spheroid culture without 2% DMSO; the ammonia concentrations increased in both cultures and the ammonia was not detoxified. But with 2% DMSO, the ammonia concentrations in the spheroid culture decreased at days 2 and 3 after inoculation on the dish coated with poly-L-glutamic acid, while the concentration in the monolayer culture still increased (Figure 9). These results showed that DMSO should be effective to induce ammonia detoxification of human fetal hepatocytes after proliferation in only spheroid culture. The averaged ammonia detoxification rate of the spheroid culture during 3 h was $0.57 \mu\text{mol}/10^6 \text{ cells/h}$, which was comparable to the rate ($0.485 \mu\text{mol}/10^6 \text{ cells/h}$) of porcine hepatocyte spheroids used in a bioartificial liver (Sakai et al. 1996).

Figure 7. Fluorescent image analysis of cytochrome P-450 activities (EROD) of human fetal hepatocytes in a monolayer and spheroids by using a laser confocal microscope. (A) Spheroid; (B) Monolayer. Area averages of fluorescence intensity of these images were 58.6 [–] in spheroids and 20.3 [–] in monolayer.

At the 1st passage, human fetal hepatocytes in the T-flask proliferated to 2.6×10^6 cells. From the 2nd passage, the cells propagated about 20-fold in one passage on a 35-mm culture dish. We considered that the total cell number would be more than

4×10^{11} cells until the 5th passage, which would be a sufficient cell number for the development of BAL.

In conclusion, proliferative human fetal hepatocytes can form spheroids on the surface of a

Figure 8. Cytochrome P450 activities of human fetal hepatocytes in a monolayer and spheroids. Values shown are averages $\pm$ S.D.

Figure 9. Ammonia metabolizing activities of human fetal hepatocytes in monolayer and spheroid cultures with 2% DMSO. (●) Monolayer culture, (○) Spheroid culture at day 2, (□) Spheroid culture at day 3.

polystyrene dish coated with negatively charged poly-L-glutamic acid or poly-L-aspartic acid. The characteristics of a suitable surface for spheroid formation of human fetal hepatocytes were different from those for adult hepatocytes. CYP activities (CYP 1A1, CYP1A2) of the cells increased about twofold in spheroid culture compared with monolayer culture after proliferation. Ammonia detoxification activity of the cells, which was lost during proliferation, recovered in spheroid culture in the presence of 2% DMSO.

These results suggest that the spheroid culture technique that uses a culture surface coated with poly-L-amino acid should be useful for BAL development that uses human fetal hepatocytes. Although the use of human fetal hepatocytes

seems to have a bioethical issue, fetal hepatocytes include hepatoblasts, which are a kind of hepatic stem cell (Grisham and Thorgeirsson 1997). Therefore, isolation and proliferation of normal hepatoblasts *in vitro*, and the use of the spheroid culture technique for hepatoblasts seems to be a future study for BAL development.

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