ORIGINAL ARTICLE

Spheroid Formation and Enhancement of Cytochrome P450 Activity for Human Fetal Hepatocytes on Poly-L-glutamic Acid Coated Dish

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SYNOPSIS
Spheroids were formed for human fetal hepatocytes on poly-L-glutamic acid-coated polystyrene dishes that had a negatively charged surface at neutral pH. The optimum concentration of poly-L-glutamic acid solution for coating the surface of the dish was 1mg/ml. The optimum cell density of inoculation for the spheroid formation was $3.4 \times 10^4$ cells/cm$^2$ ($4.0 \times 10^5$ cells/35mm-dish). The cytoskeletal actin filaments of the cells were reorganized from stress fiber in the monolayer to cortical actin in the spheroid. The spheroid formation was inhibited by cytochalasin D, which is an inhibitor of actin polymerization. Furthermore, the activity (6.6 pmol/10$^6$ nuclei/min) of cytochrome P450 1A1/2 of cells in the spheroid was almost threefold higher than that of those in the monolayer, and was comparable with that of human mature hepatocytes of primary culture. These results suggest that the reorganization of actin filaments in hepatocytes would be essential for the spheroid formation, and the spheroid culture seems to be suitable for the expression of differentiated functions of human hepatocytes.

Key words: human fetal hepatocyte, spheroid, cytochrome P450

INTRODUCTION
Mass culture and functional expression of human hepatocytes are very important subjects for the development of bioartificial liver$^{1,2}$. But primary human hepatocytes from adult liver can not proliferate in vitro. In our previous paper$^3$, it was suggested that human fetal hepatocytes could proliferate in monolayer culture, but they might loose their functions. Then, we studied the condition of three dimensional culture of human fetal hepatocytes and found that they could form spheroids on the negatively charged surface of a culture dish coated with poly-L-glutamic acid or poly-L-aspartic acid. Their functions such as cytochrome P450 activities were partially restored in spheroid culture, which were qualitatively indicated by confocal microscopy.

In this paper, we studied the optimum conditions for the spheroid formation of human fetal hepatocytes such as a concentration of poly-L-glutamic acid solution for coating the culture dish and the inoculum cell density on the dish. Furthermore, the differences in the cytoskeletal structure of cells in the monolayer and the spheroid culture and
the quantitative analysis of cytochrome P450 activities of the cells were investigated.

MATERIALS AND METHODS

1. Cell and culture medium

Human fetal hepatocytes were obtained from Dainippon Pharmaceutical Co. (Osaka, Japan) with a certification of informed consent for research. The delivered cells (1\textsuperscript{st} passage, primary culture) were serially passaged in monolayer culture with CS-C medium (Cell Systems, Kirkland, WA, USA) as described in our previous paper\cite{5}, and the 4\textsuperscript{th} passaged cells were used in all experiments. The viable cell number was measured by using the trypan blue dye exclusion method with a haemocytometer.

2. Spheroid formation of human fetal hepatocytes

Poly-L-glutamic acid (Sigma, P-4761, USA) was dissolved at various concentrations in Milli-Q water to investigate the optimum concentration for the spheroid formation. 2 ml of the solution was added to polystyrene dishes (Iwaki non-treated dish, diameter 35 mm) and was incubated at 37°C for 1 hour. The dishes coated with various concentrations of poly-L-glutamic acid were equilibrated with hormone-defined medium (HDM) for 10 min at room temperature before use. HDM comprised of Williams' medium E (Gibco, Grand Island, NY, USA) supplemented with 10 μg ml\textsuperscript{-1} insulin (Sigma, St. Louis, MO, USA), 50 ng ml\textsuperscript{-1} epidermal growth factor (Takara, Kyoto), 0.1 μM copper (CuSO\textsubscript{4}·5H\textsubscript{2}O), 3nM selenium (H\textsubscript{2}SeO\textsubscript{3}), 50 pM zinc (ZnSO\textsubscript{4}·7H\textsubscript{2}O), 50 ng ml\textsuperscript{-1} linoleic acid (Sigma), 63 μg ml\textsuperscript{-1} penicillin and 100 μg ml\textsuperscript{-1} streptomycin. HDM has been used for the spheroid formation of primary hepatocytes\cite{6}.

The 4\textsuperscript{th} passaged cells were inoculated at various cell densities in 2.0 ml of HDM in each 35 mm dish to investigate the optimum inoculum cell density for the spheroid formation. After the cultivation for 4 hours at 37°C under a humidified atmosphere of 5% CO\textsubscript{2} and 95% air, the medium was changed and thereafter that was renewed at intervals of two days. The cells were cultured for 4 days for the spheroid formation. The morphology of hepatocytes in the 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 dishes that were coated with various concentrations of poly-L-glutamic acid. The average number of spheroids and hemispheroids in each square millimeter (spheroid density) was calculated by counting their numbers on the microphotographs. Morphological estimation of spheroids and hemispheroids was according to the paper by Koide et al\cite{7}.

3. Histology

Spheroids were taken off the dish by intensive pipetting and were spun down by centrifugation at 1000 rpm for 5 min. The spheroids were fixed with 10% formalin neutral buffer solution (Wako Pure Chemical Industries, Osaka, Japan) and embedded in paraffin. A sectioned spheroid was stained with hematoxylin-eosine (HE).

To stain the actin filaments of the cells, the cells were fixed with 4% formalin neutral buffer solution (Wako) including 0.1% Triton X-100, and treated with 7.5U/ml Rhodamine phalloidin (Molecular Probes R-415 ) and 1 μM TO-PRO-3 (Molecular Probes T-3605) for 15 min at room temperature. TO-PRO-3 was used for counter-staining of cell nucleus. After washing; the stained cells were observed using confocal laser microscopy, Heidelberg,
4. Measurement of cytochrome P450 activity

Cytochrome P-450 (CYP1A1/2) activities of the cells in the monolayer and spheroid culture were specifically induced by 2 μM 3-Methylcholanthrene in HDM for 96hr culture, and were measured as ethoxyresorufin-O-dealkylation (EROD) activities 96hrs later. The measurement medium contained 20 μM ethoxyresorufin and 10 μM Dicumarol, which was an inhibitor of DT-diaphorase and was added to retard further metabolism of resorufin into non-fluorescent compounds. After the incubation at 37°C for 30min, 1.5 ml aliquot of measurement mediums in monolayer and spheroid culture were withdrawn from each dish and transferred to centrifuge tubes. 300 Fishman units of β-glucuronidase and 2400 Roy units of arylsulfatase were dissolved in 500 μL of 0.1M sodium acetate buffer, pH 4.5. The enzyme solution was added to a centrifuge tube containing 1.5 ml of the measurement medium. The centrifuge tubes were incubated at 37°C to allow hydrolysis of potential resorufin conjugates. After 2 hr, 4 ml of ethanol was added to each tube and the tubes were centrifuged at 1,800 x g for 10min. Fluorescence of supernatants was measured using a Fluorostere spec-trophotometer F-2000 (Hitachi Japan) with 530-nm excitation and 590-nm emission filters. A standard solution of resorufin was prepared with Milli-Q water. Fluorescence was linear within the range 0-100 pmol resorufin/ml. Statistical analysis was carried out using Student’s t-test. Values of p <0.05 were considered significant.

RESULTS AND DISCUSSION

As reported in our previous paper, human fetal hepatocytes formed multicellular spheroids, hemispheroid and multilayer on the culture dish. Fig.1 indicates the morphological estimation of spheroid (Fig. 1-A), hemispheroid (Fig. 1-B) and multilayer (Fig. 1-C) of human fetal hepatocytes formed on the poly-L-glutamic acid coated dish, which were

![Images of spheroid, hemi-spheroid, multilayer, and monolayer]

Fig.1 Morphological estimation of the spheroid, hemi-spheroid and multilayer of human fetal hepatocytes. Hepatocytes were cultured on the poly-L-glutamic acid coated dishes. Concentration of poly-L-glutamic acid solution for coating was 1 mg/ml.
according to the paper by Koide et al. On the basis of the estimation, optimum culture conditions for spheroid formation, such as optimum concentration of poly-L-glutamic acid solution, and optimum inoculum cell density, were investigated.

The average density of spheroids and hemispheroids in a square millimeter was estimated from microphotographs randomly taken on each dish coated with various concentrations of poly-L-glutamic acid solution (0.01~10 mg/ml) as shown in Fig. 2. Based on these results, there was no significant difference among the average densities. Consequently, the experimental condition of 1mg/ml of the poly-L-glutamic acid was selected for the spheroid formation. The average density of spheroids and hemispheroids was also estimated on each dish inoculated at various cell densities from $2.4 \times 10^4 \sim 5.1 \times 10^4$ cells/cm² ($3 \times 10^5 \sim 6 \times 10^5$ cells/35mm dish, Fig. 3). The highest density of spheroid and hemi-spheroid, 5.0 number/mm², was obtained on the dish inoculated at $3.4 \times 10^4$ cells/cm², which was almost twice as high as those...
on the dishes of $2.5 \times 10^4$ and $5.1 \times 10^4$ cells/cm². Consequently, the $3.4 \times 10^4$ cells/cm² was selected as the optimum cell density for the spheroid formation of human fetal hepatocytes.

A histological study with haematoxylin-eosin staining of a sectioned spheroid revealed that the cells in the spheroids could be viable and the spheroids of about $80 \mu$m in diameter were com-
posed of four or five cell layers, as shown in Fig. 4. Fig. 5 shows the cytoskeletal structure of actin filaments of human fetal hepatocytes in monolayer and spheroid cultures. The cells in the spheroid exhibited a more cuboidal shape along with a reorganization of cytoskeletal actin filaments from the stress fiber in the monolayer to the cortical actin in the spheroid. The cortical actin structure is well clarified in the hepatocytes of normal liver tissue in vivo. Furthermore, the spheroid formation was inhibited by cytochalasin D, which is an inhibitor of actin polymerization as shown in Fig. 6.

In our previous paper, the cytochrome P450 (CYP 1A1/2) activities in spheroid and monolayer cultures were assessed in situ using confocal laser microscopy. In this study, the enhancement of CYP 1A1/2 activity in spheroid culture was quantitatively compared with that in the monolayer culture using biochemical analysis. As shown in Fig. 7, the cells in the monolayer exhibited low average activity, even after being treated with 3-Methylcholanganthrene (3-MC), which was the inducer of CYP 1A1/2. On the other hand, the CYP 1A1/2 activity in the spheroid was almost threefold higher (p<0.01 ~ 0.02) compared with that in the monolayer with or without 3-MC. Furthermore, the activity of 6.6 pmol/10^6 nuclei/min in spheroids with 3-MC was comparable with that of human mature hepatocytes primary culture (2.5 pmol/10^6 cells/min).

The findings of this study suggest that the optimum concentration of poly-L-glutamic acid solution and optimum inoculum cell density of human fetal hepatocytes for the spheroid formation should be 1mg/ml and 3.4 x 10^4 cells/cm^2, respectively. Reorganization of actin filaments in the cells was suggested to be essential for spheroid formation, and spheroid culture seems to be suitable for the expression of differentiated function of human hepatocytes. Although the use of human fetal hepato-
toocytes for bioartificial liver seems to have a bioethical issue, fetal hepatocytes include hepatoblasts, which are a kind of hepatic stem cell. Therefore, the isolation and proliferation of normal hepatoblasts in vitro and the use of the spheroid culture technique for hepatoblasts seem to be a future study for bioartificial liver development.

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