Regular Article

Optimization of Albumin Secretion and Metabolic Activity of Cytochrome P450 1A1 of Human Hepatoblastoma HepG2 Cells in Multicellular Spheroids by Controlling Spheroid Size

Tomoko Nishikawa,^a Yutaro Tanaka,^a Makiya Nishikawa,^{*a,b} Yuka Ogino,^a Kosuke Kusamori,^a Narumi Mizuno,^a Yuya Mizukami,^a Kazunori Shimizu,^{b,c,d} Satoshi Konishi,^{b,d,e} Yuki Takahashi,^a and Yoshinobu Takakura^{a,b}

^aDepartment of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University; Sakyo-ku, Kyoto 606–8501, Japan; ^bInstitute for Innovative NanoBio Drug Discovery and Development Graduate School of Pharmaceutical Sciences, Kyoto University; Sakyo-ku, Kyoto 606–8501, Japan; ^cDepartment of Biotechnology, Graduate School of Engineering, Nagoya University; Furo-cho, Chikusa-ku, Nagoya, Aichi 464–8603, Japan; ^dDepartment of Mechanical Engineering, Ritsumeikan University; Kusatsu, Shiga 525–8577, Japan; and ^eRitsumeikan-Global Innovation Research Organization, Ritsumeikan University; Kusatsu, Shiga 525–8577, Japan. Received October 25, 2016; accepted December 20, 2016

Multicellular spheroids are useful as three-dimensional cell culture systems and for cell-based therapies. Their successful application requires an understanding of the consequences of spheroid size for cellular functions. In the present study, we prepared multicellular spheroids of different sizes using the human hepatoblastoma HepG2 cells, as hepatocytes are frequently used for *in vitro* drug screening and cell-based therapy. Precise polydimethylsiloxane-based microwells with widths of 360, 450, 560, and 770 μm were fabricated using a micromolding technique. Incubation of HepG2 cells in cell culture plates containing the microwells resulted in the formation of HepG2 spheroids with average diameters of 195, 320, 493, and 548 μm . The cell number per spheroid positively correlated with its diameter, and the viability of HepG2 cells was 94% or above for all samples. The smallest HepG2 spheroids showed the highest albumin secretion. On the other hand, the metabolic activity of 7-ethoxyresorufin, a fluorometric substrate for CYP1A1, increased with increasing spheroid size. These results indicate that controlling spheroid size is important when preparing HepG2 spheroids and that the size of HepG2 spheroids greatly influences the cellular function of HepG2 cells in the spheroids.

Key words albumin; CYP1A1; human hepatoblastoma HepG2 cell; size control; spheroid

Three-dimensional multicellular spheroids possess greater *in vivo* tissue-like morphology and function than two-dimensional monolayered cells. Therefore, multicellular spheroids are considered useful *in vitro* systems for drug screening, including tests of drug metabolism and toxicity,^{1–6)} and for studying tumor metastasis.^{7,8)} Furthermore, such spheroids have attracted much attention for application in cell-based therapy^{9–11)} and as a building block for the construction of large tissues.^{12,13)} In a previous study, we demonstrated that spheroid formation greatly prolonged the survival of insulin-secreting NIT-1 cells after transplantation in mice.⁹⁾

Spheroid size is a key parameter to determine the functions and properties of spheroids. It is reported that oxygen concentration is decreased in the core of the spheroids of human hepatoblastoma HepG2 cells, hepatocytes, and tumor cells.^{14–17)} This affects the expression of various molecules, including hypoxia inducible factor (HIF)-1. In addition, paracellular or transcellular transport is required in order for the inner cells to take up extracellular materials and to release secretory products into the culture media. Despite their importance, details regarding the relationship between spheroid size and cellular functions remain to be elucidated. In particular, few studies have investigated the effect of spheroid sizes over 300 μm on their functions and properties.

Investigating the effects of spheroid size requires the preparation of size-controlled, multicellular spheroids. Several techniques for spheroid formation have been reported,

including the spinner flask, hanging drop, and liquid overlay methods.^{18–20)} However, a common problem with these techniques is the difficulty in controlling spheroid size. In contrast, microfabrication is suitable for the development of size-controlled microwells with narrow size distribution.^{10,21,22)} In a previous study, we demonstrated that polydimethylsiloxane (PDMS)-based microwells constructed using a microfabrication technique could be used to obtain spheroids with a very narrow size distribution.²³⁾ We also found that coating the microwells with poly(*N*-isopropylacrylamide) (PNIPAAm), a thermoresponsive polymer, increased the quality of the spheroids, since the coating prevented the cells from adhering to the PDMS-based microwells.²³⁾

In the present study, we developed PDMS-based microwells of different sizes in order to prepare variably sized multicellular spheroids. HepG2 cells were used for the study as they represent one of the most extensively used cell lines to evaluate drug metabolism and toxicity.^{3,24,25)} Production of various proteins is an index of liver function. Albumin is the most abundant serum protein produced by hepatocytes.²⁶⁾ Another important liver function is the metabolism of various compounds, including drugs. CYP enzymes are the major enzymes involved in drug metabolism. CYP1A1 is one of the major CYP enzymes that is involved in the metabolic activation of aromatic hydrocarbons and is a major source of variability in drug pharmacokinetics,²⁷⁾ and its expression in HepG2 cells was reported in many studies. Therefore, albumin

* To whom correspondence should be addressed. e-mail: makiya@pharm.kyoto-u.ac.jp

and CYP1A1 were selected as indicators of cellular function.

MATERIALS AND METHODS

Chemicals Dulbecco's modified Eagle's medium (DMEM, low glucose type) was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Equitech-Bio, Inc. (Kerrville, TX, U.S.A.). A PDMS prepolymer and the curing agent (Silpot 184) were purchased from Toray-Dow Corning Co. (Tokyo, Japan). PNIPAAm was obtained from Polysciences Inc. (Warrington, PA, U.S.A.). Trypan blue stain solution (0.5%) was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Trypsin was obtained from Becton Dickinson and Co. (Mansfield, MA, U.S.A.). Ethylenediaminetetraacetic acid, disodium salt, dihydrate (EDTA-2Na) was obtained from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of the highest grade commercially available.

Cells and Cell Culture HepG2 cells were purchased from American Type Culture Collection (Manassas, VA, U.S.A.) and grown in DMEM supplemented with 10% heat-inactivated FBS, 0.15% NaHCO₃, 100 U/mL potassium penicillin G, and 100 mg/mL streptomycin sulfate at 37°C in humidified air containing 5% CO₂.

Preparation of PDMS-Based Microwells Four types of micropillar array with different widths, *i.e.*, 360, 450, 560, and 770 μ m, were fabricated as previously described. The microwells were named small, medium, large, and extra large according to their size.

PNIPAAm Coating of PDMS-Based Microwells The PDMS-based microwell sheet, within a 6-well culture plate, was coated with PNIPAAm as previously described.²³⁾

Preparation of HepG2 Spheroids HepG2 cells (5×10^6 cells for small, medium, and large microwells, or 1×10^7 cells for extra large microwells) suspended in DMEM were added to PNIPAAm-coated PDMS-based microwells, placed in 6-well culture plates, and incubated for 72 h at 37°C in a humidified 5% CO₂ incubator. The culture plates were shaken at a speed of 65 rpm for the first 3 h. Large and extra large microwells were transferred from the 6-well culture plates to a 10 cm dish after 3 h of incubation to increase the volume of culture media and to maintain high cell viability. Subsequently, these microwells were shaken at a speed of 40 rpm for an additional 69 h to enable uniform concentration of oxygen and nutrients in culture media. The culture medium was changed at 3, 24, and 48 h of incubation. To maintain the PNIPAAm coating, the coated microwells were kept at 37°C during the experiments. After 72 h incubation, the culture plates were exposed to room temperature, which caused the PNIPAAm coating to dissolve. Then, HepG2 spheroids were detached from the PDMS-based microwells and washed twice with phosphate buffered saline (PBS) to remove any remaining PNIPAAm. Thereafter, HepG2 spheroids were selected using micropipettes. Each HepG2 spheroid was named as small, medium, large, or extra large according to the PDMS-based microwells used for their preparation. HepG2 spheroids were observed under a Biozero microscope and the diameter of the spheroids was measured using the BZ analyzer software. The number and viability of HepG2 cells in one spheroid was measured by counting the cells stained with trypan blue solution after dispersion of the spheroid using a trypsin-EDTA solution (0.25% trypsin, 0.02% EDTA). To the 20 spheroids in

20 μ L of PBS was added 80 μ L of the trypsin-EDTA solution. After 5 min incubation at 37°C, the mixture was pipetted to disintegrate the spheroids into dispersed HepG2 cells.

Albumin Secretion from HepG2 Cells HepG2 spheroids containing approximately 1×10^5 HepG2 cells were added to each well of a 6-well culture plate. To achieve this, 67, 25, 12, and 7 spheroids were used for small, medium, large, and extra large HepG2 spheroids, respectively. Suspended (dispersed) HepG2 cells were prepared by detaching cells from a culture dish using a trypsin-EDTA solution (0.25% trypsin, 0.02% EDTA) and added to a well at the same number (1×10^5 cells/well). After 24 h incubation, the culture supernatants were collected and stored at -80°C until use. The concentration of human albumin in the supernatants was determined using the Human Albumin enzyme-linked immunosorbent assay (ELISA) Quantitation Set (Bethyl Laboratories Inc., Montgomery, TX, U.S.A.), according to the manufacturer's protocol.

Ethoxyresorufin-O-deethylase Assay To measure CYP1A1 activity, ethoxyresorufin-O-deethylase (EROD) assay was performed as previously reported.²⁸⁾ In brief, suspended HepG2 cells and HepG2 spheroids approximately corresponding with 8×10^4 HepG2 cells were added to a well in the 24-well plate with 10 μ M of 7-ethoxyresorufin. A number of HepG2 spheroids used in the EROD assay is 35, 20, 14, and 9 spheroids of small, medium, large, and extra large HepG2 spheroids, respectively. After 1 h incubation under lightproof condition, the fluorescence of metabolites from 7-ethoxyresorufin by HepG2 cells and spheroids was detected by FluoroMax-4 spectrofluorometer (HORIBA, Ltd., Kyoto, Japan).

Statistical Analysis Differences were statistically evaluated using one-way ANOVA followed by Tukey's test for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

Fig. 1. Microscopic Images of HepG2 Spheroids

Images of HepG2 spheroids were taken under a microscope, and typical images are shown: (A) small, (B) medium, (C) large, and (D) extra large HepG2 spheroids. Scale bar in each image represents 200 μ m.

RESULTS

Fig. 2. Size Distributions of HepG2 Spheroids

The histograms of the diameter of 100 spheroids were measured under a microscope: (A) small, (B) medium, (C) large, and (D) extra large HepG2 spheroids.

Size of HepG2 Spheroids and Viability of HepG2 Cells in the Spheroids Figures 1A–D show microscopic images of the small, medium, large, and extra large HepG2 spheroids, and Figs. 2A–D show histograms of their diameter. All HepG2 spheroids had narrow size distribution. The average diameter of the small, medium, large, and extra large HepG2 spheroids was 195, 320, 493, and 548 μm , respectively (Table 1). The number of HepG2 cells per spheroid was approximately 1500, 4000, 8500, and 13000 for the small, medium, large, and extra large HepG2 spheroids, respectively (Table 1). Cell viability was 94% or greater for all spheroids. Cell viability of monolayered HepG2 cells treated with the trypsin–EDTA solution was 98.5% or more (data not shown), suggesting that the treatment of HepG2 spheroids with the trypsin–EDTA solution hardly affected the viability of the cells and spheroid formation slightly reduced it.

Secretion of Albumin from Monolayered HepG2 Cells and HepG2 Spheroids We measured the concentration of albumin in the culture medium of monolayered HepG2

Table 1. Characteristics of HepG2 Spheroids

	Diameter (μm)	Cell number	Viability (%)
Small	195 $\pm$ 27	1500 $\pm$ 220	95.4 $\pm$ 2.9
Medium	320 $\pm$ 21	4080 $\pm$ 360	97.6 $\pm$ 1.7
Large	493 $\pm$ 32	8450 $\pm$ 510	96.5 $\pm$ 1.7
Extra large	548 $\pm$ 33	13000 $\pm$ 1300	94.1 $\pm$ 1.1

Diameter is expressed as the mean $\pm$ S.D. of 100 measurements. Cell number and viability are expressed as the mean $\pm$ S.D. of five measurements.

Fig. 3. Cellular Functions of Monolayered HepG2 Cells and HepG2 Spheroids: Albumin Secretion (A) and EROD Activity (B) of HepG2 Cells

(A) Monolayered HepG2 cells (suspended before use) or HepG2 spheroids were added to each well of a 6-well culture plate at approximately 1×10^5 cells/well. The concentration of albumin in culture media was measured 24 h after incubation. Results are expressed as the mean $\pm$ S.D. of five measurements. A representative experiment of three independent experiments with similar results is presented. (B) 8×10^4 monolayered HepG2 cells and HepG2 spheroids which correspond with 8×10^4 HepG2 cells were incubated with $10 \mu\text{M}$ of 7-ethoxyresorufin for 1 h. After the incubation, the supernatants were used for measurement of fluorescence intensity with FluoroMax-4. Results are expressed as the mean $\pm$ S.D. of three measurements. A representative experiment of five independent experiments with similar results is presented. * $p < 0.05$ versus the monolayer group.

cells and HepG2 spheroids (Fig. 3A). The small and medium HepG2 spheroids released significantly higher amounts of albumin than the monolayered HepG2 cells, and large and extra large HepG2 spheroids.

EROD Assay of Monolayered HepG2 Cells and HepG2 Spheroids The activity of isozyme CYP1A1 was measured by the EROD assay (Fig. 3B). The activity elevated with increasing spheroid size. The activity of the large and extra large HepG2 spheroids was significantly higher than that of monolayered HepG2 cells.

DISCUSSION

The method based on PDMS-based microwells developed in the previous study is superior to other techniques in terms of productivity and uniformity in size.²³⁾ The present study clearly demonstrates that PDMS-based microwells can effectively prepare different sized multicellular spheroids, with a narrow size distribution (Figs. 1, 2, Table 1).

Previous reports have discussed the relationship between spheroid size and cellular functions. For instance, it was shown that albumin secretion from HepG2 spheroids with diameters between 125 and 250 μm increased as spheroid size increased.²⁴⁾ On the other hand, levels of secretion of α -fetoprotein, albumin, and fibrinogen in HepG2 spheroids, with diameters ranging between 100 and 250 μm , was independent of spheroid size.²⁵⁾ However, little is known about the properties of HepG2 spheroids larger than 250 μm .

It was reported that large HepG2 spheroids with diameters of 180 μm or greater contain dead cells in their central regions because of minimal or no transport of oxygen and nutrients.^{24,29)} These reports are inconsistent with our results, which demonstrate the high viability of large and extra large HepG2 spheroids (94% or above). The increased volume of culture media added to large and extra large microwells could explain this. Supporting this hypothesis, when large and extra large HepG2 spheroids were incubated for 72 h in 6-well culture plates, as were the small and medium HepG2 spheroids, the average diameter of the large and extra large HepG2 spheroids was smaller (361 and 517 μm , respectively) than those used for this experiment (493 and 548 μm ; Table 1). Furthermore, the viability of large and extra large HepG2 spheroids was lower (74.3 and 65.9%, respectively) than those used (97 and 94%). In addition, it has been reported that increasing the glucose concentration of the culture media reduces the number of dead cells in the spheroids of EMT6/Ro mouse mammary tumor cells and MGH-U1 human bladder cancer cells.^{17,30)} Therefore, it can be speculated that the viability of large and extra large HepG2 spheroids was high in this experiment because the cells in the spheroids were supplied with sufficient nutrients during the culture period.

Previous studies have shown that the cellular functions of HepG2 cells and hepatocytes are improved by spheroid formation because of cell–cell interactions.^{1,2,23,31)} The extensive cell–cell interaction could explain the enhanced albumin secretion and EROD activity in HepG2 cells when formulated in multicellular spheroids.

Protein synthesis and metabolism are two major functions of hepatocytes. In the present study, albumin secretion and CYP1A1 activity were selected as indicators to evaluate the function of HepG2 spheroids, because they can be measured

with high sensitivity. Numerous reports have indicated that a low concentration of amino acids directly reduces the rate of albumin synthesis both in intact animals and in isolated hepatocytes.^{32,33)} Similarly, the rate of albumin synthesis of rats fed with protein free or low-protein diets for 2–3 weeks was lower than that of rats fed with normal diets; moreover, decreased albumin secretion was also observed in isolated rat hepatocytes.^{34–37)} On the other hand, previous studies reported that the formation of spheroids using human fetal hepatocytes and human hepatocellular carcinoma cells, including HepG2 cells, resulted in higher expression of CYP1A1 proteins and genes, respectively, compared to the monolayered cells.^{38,39)} Moreover, it has been reported that the exposure of male Sprague-Dawley (SD) rats to hypoxic conditions for 48 h increased the EROD activity in the liver.⁴⁰⁾ As the size of HepG2 spheroids became larger, hypoxic areas in HepG2 spheroids will increase, because the core region of multicellular spheroids is known to be under hypoxic conditions. Consequently, it is likely that the elevation in the EROD activity in the present study is attributed to the spheroid size-dependent expansion of hypoxic areas in the spheroids.

In conclusion, we successfully developed HepG2 spheroids with precisely controlled size using precise PDMS-based microwells with widths of 360, 450, 560, and 770 μm . We found that the spheroid formation generally improved the function of HepG2 cells, but the spheroid size differently affected the albumin secretion and CYP1A1 activity of HepG2 cells. These results indicate that controlling spheroid size is important and that the size of HepG2 spheroids greatly influences the cellular function of HepG2 cells in the HepG2 spheroids.

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Conflict of Interest The authors declare no conflict of interest.

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