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Micropatterned Organoid Culture of Rat Hepatocytes and HepG2 Cells

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The culture of liver cell organoids (multicellular aggregates) such as spheroids or cylindroids, which can strongly express liver functions, has been advocated as a useful technique that has advantages over monolayer culture. This paper describes a micropatterning technique for obtaining spheroids and cylindroids by using rat hepatocytes or HepG2 cells. We developed culture chips that comprised multiple, circular or rectangular microwells; the bottom surface of each microwell was modified with collagen to create a cell adhesion area, and the entire microwell, excluding the collagen-coated spots, was modified with polyethylene glycol (PEG) to create a nonadhesive area. Rat hepatocytes and HepG2 cells formed uniform spheroids and cylindroids on the circular and rectangular chips, respectively. Consequently, two-dimensional micropatterned chips containing homogeneous spheroids or cylindroids were generated. The expression of liver functions (protein secretion and ammonia removal) was greater in the spheroids and cylindroids than in the monolayer culture, and this expression was maintained for at least 2 weeks of culture. Thus, this chip technology has potential for use in various applications that involve organoid culture.

[**Key words:** rat hepatocyte, HepG2 cell, organoid, spheroid, cylindroid, micropatterning, chip]

The liver plays many essential roles in maintaining the normal physiology. Therefore, highly functional liver cells such as primary hepatocytes and HepG2 cells have been used for various purposes such as pharmacological and toxicological studies, bioartificial liver construction, and fundamental cell biology studies (1–11). For the success of such applications, high expression and long-term maintenance of liver cell functions in cultures are important.

It is generally known that the functions of monolayer liver cell cultures, which exhibit a spreading morphology on the culture dish, are reduced or lost during the culture (4–6). In contrast, liver cell organoids such as spheroids, which are spherical multicellular aggregates (12–15), and cylindroids, which are cylindrical multicellular aggregates (16, 17), can maintain a high level of liver function *in vitro*; this is because they resemble tissues in terms of the presence of abundant cytoplasmic organelles and close cell–cell contact (18–20). Therefore, organoid culture has been advocated as a useful technique to replace monolayer culture. Here, the cylindroid culture has some advantages than spheroid culture: (i) it can achieve a high cell density culture of organoid per unit area of culture substratum, and (ii) it can form large tissue-like structure than spheroid, because it consists of cylindrical structure. However, it is difficult to control the size, location, and immobilization of organoids on the culture scaffolds by using previously reported organoid techniques such as rotational culture or in a culture dish that promotes organoid formation. On the other hand, some researchers have reported techniques for hepatocyte spheroid micropatterning via surface modifications or microfabrication of culture scaffolds (21–23). We have previously developed micropatterned chips comprising rat hepatocyte spheroids or HepG2 spheroids by using soft lithography or microfabrication techniques (24–26). Although some spheroid micropatterning techniques have been established as mentioned above, no study has been conducted to investigate micropatterning techniques for organoids other than spheroids, for example, cylindroids. Furthermore, clarification of the relationship between the shape of the organoid and the expression of liver functions may provide useful information for the study of organoid culture.

In the present study, we developed organoid chips comprising micropatterned uniform spheroids or cylindroids of rat hepatocytes or HepG2 cells. Furthermore, the features of organoids were clarified by comparing the expression of liver functions between spheroid and cylindroid cultures.

MATERIALS AND METHODS

Microfabrication of organoid chips Figure 1 shows a schematic diagram of the 2 types of organoid chips used, namely, a spheroid chip and a cylindroid chip. We previously reported that the diameters at the survival limit of rat hepatocyte spheroids and HepG2 spheroids were approximately within 150 and 200 μm, respectively (24–26). Therefore, both the chips were designed to form organoids of less than approximately 200 μm in diameter.

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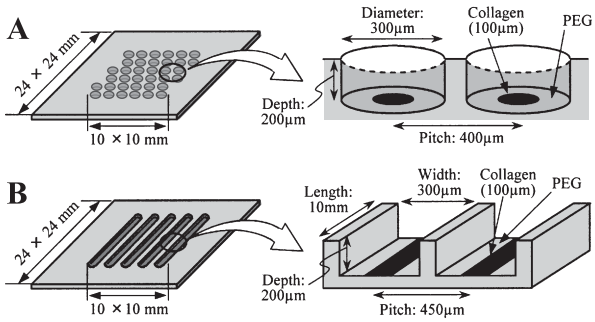

FIG. 1. Schematic diagram of a spheroid chip (A) and a cylindroid chip (B).

The spheroid chip comprised 672 circular microwells in a triangular arrangement (pitch, 400 μm) on a poly(methylmethacrylate) (PMMA) plate (24×24 mm); each circular microwell was 300 μm in diameter and 200 μm in depth (Fig. 1A). In the center of the bottom surface of each circular microwell, an area with a diameter of 100 μm was modified with collagen to create a cell adhesion area; further, the entire microwell, excluding the collagen-coated spots, was modified with polyethylene glycol (PEG) to create a nonadhesive area. This chip was prepared using the microfabrication and microcontact printing techniques (24). The spheroid chip substrate comprised 2 PMMA plates. On one of the plates that had a thickness of 200 μm, holes with a diameter of 300 μm were bored using a programmable micromilling system (PMT, Fukuoka). To configure the culture microwells, this plate was placed on the other raw PMMA plate that had a thickness of 200 μm, and the plates were bonded by press heating at 107°C for 4 h. The surface of the chip was then coated with a 6-nm-thick layer of platinum in an ion sputter unit (Hitachi Science Systems, Ibaragi). For microcontact printing, a stamp was molded by casting the liquid prepolymer of poly(dimethylsiloxane) (PDMS; Sylgard 184; Dow Corning, Midland, MI, USA) over the stamp master, which comprised a PMMA plate containing small concave wells with a head diameter of 100 μm. The PDMS stamp was briefly oxidized in air plasma (Harrick Scientific, Ossining, NY, USA) and inked with a 0.15% collagen type I solution (Cellmatrix; Nitta Gelatin, Osaka). The inked stamp was microscopically brought into contact with the center of the bottom surface of the microwells to create a cell adhesion area and then carefully peeled off from the chip substrate. Next, the chip was immersed in ethanol solution supplemented with 5 mM PEG containing a thiol group (PEG-SH; molecular weight, 10,000) (NOF, Tokyo) to obtain a nonadhesive area around the collagen-coated spots. By using this procedure, PEG was covalently attached to the platinum-coated surface via the thiol group. The collagen/PEG-micropatterned spheroid chip was thoroughly rinsed with distilled water and then in 50% ethanol for sterilization and removal of the unbound PEG-SH. The chip was then immersed in the culture medium until use.

The cylindroid chip comprised a PMMA plate (24×24 mm) containing 20 rectangular microwells (pitch, 450 μm), each of which was 300 μm in width, 10 mm in length, and 200 μm in depth (Fig. 1B). In the center of the bottom surface of each rectangular microwell, an area with a width of 100 μm and a length of 9.2 mm was modified with collagen to create a cell adhesion area; the entire microwell, excluding the collagen-coated spots, was then modified with PEG to create a nonadhesive area. The cylindroid chip was prepared using the same procedures that were used for the spheroid chip, except that the microwells were rectangular in the former.

Rat hepatocyte culture Rat hepatocytes were isolated from the whole liver of an adult Wistar rat (male, 7–8 weeks old and weighing approximately 200 g) by performing liver perfusion using 0.05% collagenase (Wako Pure Chemical Industries, Osaka). The cell viability was determined using the trypan blue exclusion method, and cells exhibiting more than 85% viability were used for the subsequent analyses. The culture medium comprised Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 μg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA), 7.5 μg/ml hydrocortisone (Wako Pure Chemical Industries), 50 ng/ml epidermal growth factor (Biomedical Technologies, Stoughton, MA, USA), 60 mg/*l* proline (Wako Pure Chemical Industries), 50 ng/ml linoleic acid (Sigma), 0.1 μM $CuSO_4 \cdot 5H_2O$, 3 ng/ml H_2SeO_3, 50 pM $ZnSO_4 \cdot 7H_2O$, 58.8 μg/ml penicillin, and 100 μg/ml streptomycin.

Hepatocytes (1.7×10^5) were inoculated onto the spheroid or cylindroid chip placed in a polystyrene dish (diameter, 35 mm) containing 2 ml culture medium. This cell density was chosen to form organoids of approximately 150 μm in diameter. After 4 h of culture, the cell-inoculated chip was transferred to another polystyrene dish containing 2 ml fresh culture medium to remove the cells that had not adhered to the chip. To obtain a fine monolayer of cells as the control group, hepatocytes (3.0×10^5) were inoculated onto a 35-mm dish coated with type I collagen (Asahi Techno Glass, Tokyo). The culture medium was changed at intervals of 1 d. All the cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

HepG2 cell culture HepG2 cells (RCB1648; Riken Cell Bank, RIKEN BioResource Center, Ibaraki) were cultured as a continuous monolayer in a 55-cm^2 tissue culture dish (Corning, Corning, NY, USA) containing 13 ml Williams' medium E (Sigma) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

HepG2 cell suspension was obtained by treating the confluent monolayer formed on the tissue culture dish with 0.25% trypsin. HepG2 cells (1.0×10^5) were inoculated onto the spheroid or cylindroid chip placed in a polystyrene dish (diameter, 35 mm) containing 2 ml culture medium. In a preliminary study, this cell density was found to be optimum for cell attachment onto the collagen-coated areas of both chips. After 4 h of culture, the cell-inoculated chip was transferred to another polystyrene dish containing 2 ml fresh culture medium to remove the nonadherent cells. To obtain the usual monolayer of cells as the control group, HepG2 cells (2.0×10^5) were inoculated onto a 35-mm tissue culture dish (Falcon 3001; Becton Dickinson, Lincoln Park, NJ, USA). The culture medium was changed at intervals of 1 d. All the cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Organoid diameter measurement To evaluate the changes in the spheroid or cylindroid diameter with time, images of the spheroids or cylindroids formed under each condition were captured at 3, 5, 7, 10, and 14 d of culture by using a phase-contrast microscope. The organoid diameters were measured using a Windows personal computer installed with a two-dimensional image analysis program (WinROOF; Mitani, Fukui). The spheroid and cylindroid diameters were calculated using the equivalent circle diameter method and the two-point distance method, respectively. The spheroid and cylindroid diameter data are expressed as the mean ± standard deviation (SD) of 100 spheroids and 100 points of cylindroids, respectively.

Liver functions Albumin secretion and ammonia removal in the rat hepatocyte culture and albumin and alpha-fetoprotein secretion in the HepG2 cell culture were evaluated as typical liver functions. The concentrations of rat or human albumin and human alpha-fetoprotein, which were secreted into the culture medium during 24 h of culture, were determined by performing an enzyme-linked immunosorbent assay (ELISA). To evaluate ammonia removal in the rat hepatocyte culture, the culture medium was replaced with a fresh culture medium containing 1 mM ammonium chloride. The ammonia concentration was measured using a commercial kit (Wako Pure Chemical Industries). The ammonia removal activity