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Changes in HepG2 spheroid behavior induced by differences in the gap distance between spheroids in a micropatterned culture system

Daisuke Miyamoto, Takuya Hara, Ai Hyakutake, and Kohji Nakazawa*

Department of Life and Environment Engineering, The University of Kitakyushu, 1-1 Hibikino, Wakamatsu-ku, Kitakyushu, Fukuoka 808-0135, Japan

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Micropatterning is a promising technique for modulating culture environments. In this study, we investigated the effect of spheroid separation distance on their properties in a micropatterned chip of HepG2 spheroids. The basic chip design consisted of 37 collagen spots (300 μm in diameter) in a hexagonal arrangement on a glass substrate; the region without collagen-spots was modified by polyethylene glycol to create the non-adhesive surface. Three similar chips were fabricated with gap distances between collagen-spots of 500, 1000, and 1500 μm. HepG2 cells adhered on the collagen spots and then formed spheroids via cell proliferation. Although the albumin secretion activities of HepG2 spheroids were almost the same in all chips, inhibition of spheroid growth and anaerobic metabolism were intensified when the gap distance was less than 1000 μm. Additionally, such phenomena which are induced by interference effects between spheroids, were more pronounced at the inside region of the chip than at the outside region. However, the interference effect between spheroids was nearly avoided when the gap distance was at least 1500 μm. Furthermore, the concentration of dissolved oxygen between neighboring spheroids decreased as the gap distance decreased, indicating that the spheroids competed for oxygen and became hypoxic in a way that depended on the spheroid separation distance. These results indicate that the spheroid separation distance is an important factor that can modulate the spheroid properties.

[**Key words:** HepG2 spheroid; Micropatterned culture; Cell proliferation; Albumin secretion; Oxygen concentration]

Spherical multi-cellular aggregates (spheroids), which are formed by the rearrangement and compaction of cell aggregates, have been utilized as an *in vitro* tissue model for cancer research, or building blocks for *in vitro* tissue construction (1–3). Furthermore, spheroids derived from embryonic stem (ES) cells, termed embryoid bodies, or from induced pluripotent stem (iPS) cells have been widely used as a trigger for the *in vitro* differentiation of stem cells (4–6). Thus, the spheroid culture is a promising technique for tissue engineering research, pharmacological and toxicological studies, and fundamental studies in cell biology.

Because the spheroids are cell clumps, the inside and the outside of the spheroid are characterized by distinct biochemical interactions and parameters. Inside the spheroid, multi-cellular interactions, mediated by cell–cell contact and mechanical stress, and concentration gradients of various substances (oxygen, nutrients, and wastes) occur (7–11). For example, it is known that oxygen concentration within a spheroid gradually decreases from the periphery to the center (12–15). These parameters vary with the spheroid size, and consequently, differences in spheroid size affect the growth and function of spheroids (16–19). Therefore, the control of spheroid size is important for the regulation of spheroid properties.

On the other hand, at the outside of the spheroid, spheroid–spheroid interactions, a sign of interference between neighboring spheroids, may occur when many spheroids exist in the same culture system. The occurrence of such phenomena is supported by our previous study, which also show that the differentiation fates of ES cells varied with the distance between neighboring spheroids (embryoid bodies) (20). However, to our knowledge, there have been few reports regarding the interference between neighboring spheroids. Here, the oxygen environment surrounding spheroids may be one of key factors which induce the spheroid–spheroid interactions, because the solubility of oxygen in the culture medium is very low. Therefore, in this study, we established a measuring technique of the oxygen level of spheroid neighborhood in a micropatterned culture and evaluated the effect of oxygen environment on the spheroid behaviors. This approach will provide information useful for designing of spheroid culture including development of a spheroid microarray.

Recent soft lithography techniques, such as microcontact printing and microstencils, allow segregating cell adhesion and non-adhesion areas on the surface of a culture substratum (21,22). On such a culture substratum, where these areas are micropatterned, cells first form a monolayer on the adhesion spots and then form spheroids via cell proliferation; consequently, we can easily control the distance between neighboring spheroids (17,18,20). Therefore, among various culture techniques, cell culture in such micropatterned surfaces is a promising method to evaluate spheroid–spheroid interactions.

In this study, we focused on the relationship between spheroid behavior and spheroid separation distance. We designed spheroid

* Corresponding author. Tel.: +81 93 695-3292; fax: +81 93 695-3359.
E-mail address: nakazawa@kitakyu-u.ac.jp (K. Nakazawa).

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micropatterned chips where the distances between neighboring spheroids were 500, 1000 and 1500 μm, and then compared the proliferation, functionality, and basic metabolism of HepG2 spheroids on these chips. Furthermore, the distribution of oxygen concentration and the expression of hypoxia gene markers were evaluated to gain further insight regarding spheroid–spheroid interactions.

MATERIALS AND METHODS

Spheroid micropatterned chips The spheroid micropatterned chip contained 37 collagen spots (300 μm in diameter) in a hexagonal arrangement on a glass substrate (24 × 24 mm) that served as the cell adhesion area; the region outside the collagen spots was modified with polyethylene glycol (PEG) to form the non-adhesive area. Three similar chips with different gap sizes, namely, 500 μm (gap 500), 1000 μm (gap 1000), and 1500 μm (gap 1500), were designed to investigate the effect of the separation distance between the spheroids (Fig. S1).

The chip was fabricated by microcontact printing (17,20,23). First, the surface of the glass substrate was coated with a 6-nm-thick layer of platinum using an ion sputtering unit (Hitachi High-Tech Science Systems, Japan). A micro-stamp 300 μm in diameter to be used for the microcontact printing was molded by casting the liquid prepolymer (poly(dimethylsiloxane); PDMS; Sylgard 184; Dow Corning Co., Midland, MI, USA). The PDMS-stamp was inked with a 0.1% collagen solution (Nitta Gelatin, Osaka, Japan) and was brought into contact with the center of the chip to create the cell adhesion area. Here, collagen was adopted as better matrix of HepG2 cell immobilization (17). Next, the chip was immersed in 2.5 mM PEG carrying a thiol group (PEG-SH; molecular weight, 30,000; NOF, Tokyo, Japan) in an ethanol solution to create the non-adhesive area around the collagen spots. The chip was rinsed in 50% ethanol for sterilization and the removal of unbound PEG-SH, and then was immersed in culture medium until use.

HepG2 cell culture HepG2 cells (RCB1648) were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The cells were cultured as a uniform monolayer in a 55-cm^2 tissue culture dish (Corning Inc., NY, USA) containing 10 mL Williams' medium E (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin.

HepG2 cells grown to confluence in the tissue culture dish were trypsinized (0.25% trypsin; Wako, Japan) and resuspended in culture medium. Subsequently, 5×10^5 cells were seeded onto the chip that was placed in a polystyrene dish (35 mm in diameter). After approximately 4 h in culture, the cell-seeded chip was transferred to another polystyrene dish containing 2 mL of fresh culture medium in order to remove the cells that did not adhere to the collagen spots. By this procedure, approximately 370 cells adhered on each collagen spot. The culture medium was changed every other day. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

Measurement of spheroid size To evaluate changes in spheroid size over time, images of spheroids formed on the micropatterned chip were obtained using a phase-contrast microscope. The spheroid size was measured using a 2-dimensional image analysis program (Win ROOF; Mitani, Tokyo, Japan). The size was calculated by the equivalent circle diameter (ECD) method whereby the spheroid area was converted into an ECD.

Measurement of cell number The cell number which adhered to the collagen spot was determined by nucleus staining method. After 4 h in culture, the cell-seeded chip was fixed with a solution of 2.5% glutaraldehyde and 4.0% paraformaldehyde at room temperature for 10 min. The fixed samples were permeabilized with 0.1% Triton-X for 5 min and blocked in PBS buffer containing 1% bovine serum albumin for 20 min. The samples were then incubated with 1 μg/mL 4,6-diamidino-2-phenylindole (DAPI, Dojindo Laboratories, Kumamoto, Japan) in PBS buffer for 20 min to stain the nucleus. All procedures were done under room temperature. The stained samples were observed using a fluorescence microscope (Biorevo BZ-9000; Keyence Corporation, Osaka, Japan) (Fig. S2), and the cell number was determined by the nucleus-counting.

Cell numbers were determined after 14 d in culture using a modified DNA-DAPI fluorescence method (24). Briefly, DNA extracted from HepG2 spheroids was mixed with DAPI at a 1:1 ratio, and the fluorescence was measured with a fluorescence plate reader (excitation wavelength: 355 nm; emission wavelength: 460 nm) (Fluoroskan Ascent; Thermo Electron Corp., Finland). A standard curve for the DNA content was prepared using suspensions of known cell numbers, and was used to convert the DNA-DAPI fluorescence values into cell numbers.

Albumin, glucose, and lactate assays The albumin secretion activity of HepG2 spheroids in each chip was evaluated as a cell function index. The concentration of albumin in the culture medium was determined by enzyme-linked immunosorbent assay (ELISA). Glucose consumption and lactate production were evaluated as basal metabolism indices. The concentrations of glucose and lactate in the culture medium were measured using a Glucose Test Wako (Wako) and Lactate Assay Kit II (BioVision Inc., Milpitas, CA, USA), respectively. These values were normalized against the cell numbers estimated on the same day that the medium was collected for albumin, glucose, and lactate measurements.

Real-time polymerase chain reaction analysis The expression of typical gene markers of HepG2 spheroids, such as albumin (*ALB*) as a hepatic functional marker, glucose transporter 1 (*GLUT1*) and lactate dehydrogenase A (*LDH1*) as basal metabolism markers, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a housekeeping gene, were analyzed by real-time polymerase chain reaction (PCR).

The spheroids after 14 d of culture were scraped off from the chip with a spatula, and were collected for gene expression analysis. Total RNA was extracted from each sample using a spin column (NucleoSpin RNA II; Nippon Genetics, Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized from 0.2 μg total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Japan). The samples were stored at −20°C until they were processed for real-time PCR analysis.

Real-time PCR was performed on an Applied Biosystems StepOne Real-Time PCR system using TaqMan Gene Expression Assay Kits (*ALB*, Hs00609411_m1; *GLUT1*, Hs00892681_m1; *LDH1*, Hs00855332_g1; *GAPDH*, Hs02758991_g1; Applied Biosystems). The comparative cycle time (ΔΔCT) method was used to quantify gene expression according to the manufacturer's protocol. The expression levels of the target genes were normalized to that of *GAPDH*. Using the gene expression levels in the initially prepared HepG2 cells as a reference, the gene expression levels in other samples were calculated (the expression levels of the initially prepared HepG2 cells were set to 1).

Measurement of oxygen concentration The oxygen concentration in the culture medium containing the spheroids was measured by a needle-type oxygen microsensor (Microx TX3; PreSens-Precision Sensing GmbH, Regensburg, Germany). The sensor probe was set to the vicinity of the spheroids in the culture medium, and the oxygen concentration was determined at 37°C under a humidified atmosphere of 5% CO_2.

Statistical analysis Data are presented as mean values ± standard deviation (SD). All experiments were performed at least three times and showed reliable reproducibility. Statistical differences in cell number, albumin, glucose, and lactate levels between different conditions were evaluated by a repeated measures analysis of variance. The statistical analysis of gene expression was performed using a *t*-test. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Formation and growth of HepG2 spheroids HepG2 cells seeded on the chip adhered to the collagen spots within 4 h and formed a monolayer after 1 d in culture. After approximately 3 d in culture, the cells formed a spheroid on each collagen spot, and the spheroids grew via cell proliferation (Fig. 1).

Although the spheroid size increased on each collagen spot, the growth differed depending on the location of the spheroid on the chip. To clarify this effect, the spheroids were classified according to their chip location (Fig. 2A). The outside spheroids consisted of 18 spheroids that were located on the outer hexagonal ring of the spots, whereas the inside spheroids consisted of 19 spheroids that were located in the center region, covering all spots in the area surrounded by the hexagonal ring. In the gap 500 chip, spheroids from both regions grew to almost the same size after 3 d in culture, but after 5 d in culture the increase in size was higher for the spheroids in the outside region than for those the inside region and the difference in the size between spheroids from the two regions increased with increasing culture time (Fig. 2B). A similar tendency was also observed in the gap 1000 chip (Fig. 2C). In contrast, in the gap 1500 chip, the increase in the size of spheroid from both regions was almost the same until 10 d of culture; subsequently, a slight size difference appeared (Fig. 2D).

To gain further insights regarding spheroid growth, the morphology and size distributions of spheroids, as well as the cell number contained within spheroids, were compared between all conditions after 14 d in culture (Fig. 3). The average spheroid size in the gap 500, 1000, 1500 chips was approximately 360, 500, and 570 μm, respectively, but their size distribution greatly depended on the distance between neighboring spheroids. In the gap 500 and 1000 chips, there were marked differences in the size of spheroids from the outside and inside regions; in contrast, in the gap 1500 chip there was hardly any difference in the size of spheroids of the

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