Preparation of arrays of cell spheroids and spheroid-monolayer cocultures within a microfluidic device

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ABSTRACT

This study describes a novel method for generation of an array of three-dimensional (3D) multicellular spheroids within a microchannel in patterned cultures containing one or multiple cell types. This method uses a unique property of a cross-linked albumin coated surface in which the surface can be switched from non-adhesive to cell adhesive upon electrostatic adsorption of a polycation. Introduction of a solution containing albumin and a cross-linking agent into a microchannel with an array of microwells caused the entire surface, with the exception of the interior of the microwells, to become coated with the cross-linked albumin layer. Cells that were seeded within the microchannel did not adhere to the surface of the microchannel and became entrapped in the microwells. HepG2 cells seeded in the microwells formed 3D spheroids with controlled sizes and shapes depending upon the dimensions of the microwells. When the albumin coated surface was subsequently exposed to an aqueous solution containing poly(ethyleneimine) (PEI), adhesion of secondary cells and fibroblasts occurred in the regions surrounding the arrayed spheroids. This coculture system can be coupled with spatially controlled fluids such as gradients and focused flow generators for various biological and tissue engineering applications.

INTRODUCTION

There has been a great deal of interest in cell-based microfluidic systems for the development of culture conditions and analysis methodologies in diverse fields ranging from fundamental biological studies to drug discovery (1-3). Microfluidics technologies are highly advantageous in a number of situations that require miniaturization and precise control of fluid elements. For example, gradient and focusing flow generators have been used to control the spatial distributions of soluble factors to study cell behaviors such as migration, growth, and neurite elongation (4-6). The focusing flow generators that form hydrodynamic two-dimensional (2D) sheath flow have also been employed to generate precisely controlled microenvironments at the sub-cellular level (7, 8). Relative to this significant progress in control over soluble factors, technologies for controlling others aspects of the cellular microenvironment in fluidic channels has been lagging.

Cell behavior in a microfluidic system has often been evaluated on a 2D flat surface for an individual cell type (9). While some cells types, such as fibroblasts and endothelial cells, are capable of proliferation and retain their function on a flat substrate, many cell types such as hepatocytes and pancreatic cells frequently lose their organ-specific functions in 2D culture and require 3D culture conditions to maintain

such functions (10, 11). Furthermore, *in vivo,* cells are surrounded by other cell types and interact with each other, influencing their respective functions. Thus, approaches that spatially localize one or more cell types are currently being intensively investigated (12, 13). Given these facts, cocultures that are suited for each cell type, such as the combination of 3D and 2D cultures, in a microchannel may be of benefit for enhancing the efficacy of assays and to generate more sophisticated cell-based microfluidic assays.

Several coculture systems having combinations of 2D and 3D cultures have been reported in stationary culture and have demonstrated the importance of these culture modes and heterotypic cell-cell interactions (14-16). Examples of 3D spheroid cultures with one cell type in a microchannel have also been prepared; these have hydrogel microstructures such as photocrosslinkable poly(ethyleneglycol) (17). Although these systems allow for 3D culture of a single cell type in a microchannel, they may not be suitable for coculture of additional cell types because of the non-adhesive property of the hydrogels.

In this paper, we present a novel method for fabricating 2D and 3D cocultures in a microchannel. We take advantage of unique characteristics of the cross-linked albumin coated surface for this purpose. The surface initially repels cells and can be switched to become cell adhesive to facilitate 3D spheroid formation and subsequent

formation of patterned cocultures. We further describe the coupling of the developed coculture system with the gradient and focusing flow generators.

MATERIALS AND METHODS

Materials and reagents

Swiss 3T3 murine fibroblasts (RCB1642), hepatoblastoma cells (HepG2, RCB1618), pheochromocytoma cells (PC12, RCB0009) were purchased from Riken Cell Bank, Japan. The reagents used for cell culture were purchased from the following commercial sources: Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) from Invitrogen, USA; bovine serum albumin (BSA) from Sigma, USA; ethylene glycol diglycidyl ether (EGDE), 50% PEI aqueous solution, fluorescein diacetate, and ethidium bromide from Wako, Japan. The materials used for fabricating culture substrates were purchased from the following commercial sources: negative photoresist SU-8 2050 from Microchem, USA; and poly(dimethylsiloxane) (PDMS) from Shinetsu silicone, Japan. All other chemicals were purchased from Sigma, unless otherwise indicated.

Cell preparation

Fibroblasts and HepG2 cells were maintained in high-glucose DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin and passaged every 3 and 5 days, respectively. PC12 cells were maintained in low-glucose DMEM containing 15% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin and passaged every 5 days. The media for each culture were changed every other day. All cells were maintained at 37 ºC, 5% CO_2 in a humidified incubator.

Device fabrication

The microfluidic device shown in Fig. 1A is composed of 3 PDMS substrates. The PDMS structures were fabricated through a replica molding process by using photolithography with negative photoresist (SU-8 2050). A PDMS replica was molded against a master by casting a liquid prepolymer PDMS solution composed of a mixture of 10:1 silicone elastomers and a curing agent. The mixture was cured at 80 ºC for 30 minutes and the PDMS replica was then peeled off from the master. The 3 PDMS substrates were irreversibly bonded to form microchannels by using an oxygen plasma treatment. After the bonding process, the microdevice was left at room temperature for 2 days to generate a sufficiently hydrophobic surface of the microchannels prior to use. The 5 inlets to the microchannels were connected to a multi-syringe pump (As One

Corp., Japan) via silicon tubes.

Microchannels in the upper PDMS were designed for trapping and removing air bubbles. The microchannels in the middle PDMS consist of a branched channel for generating a concentration gradient, 2 side channels for cell seeding or a formation of focusing flow, and a main channel for cell culture. The height of all channels formed in the middle PDMS was 50 μm, and the widths of the branched channel, the side channel, and the main channel were 100 μm, 200 μm, and 1000 μm, respectively. The lower PDMS was used to generate an array of microwells in the bottom surface of the main channel (Fig. 1A). The microwells were 100 μm in diameter and 70 μm in depth. The array of microwells contained 250 individual wells spaced evenly from each other.

3D spheroid microarray and cocultures

The preparation procedures are schematically shown in Fig. 1B. A solution containing 20 mg/ml albumin, 215 mM EGDE, and 0.5% glycerol was prepared and introduced into the main channel from the outlet. The solution was in contact with the entire surface of the main channel with the exception of the interior of the microwells because air bubbles were trapped in the microwells. After 2 h incubation at room temperature, the solution was aspirated through the outlet and dried overnight. Then

70% ethanol was introduced into the microchannels for sterilization and air bubbles (including those within the microwells) were removed by physical stimuli. The microchannels were washed with PBS, which was replaced with the culture medium.

Primary cells (HepG2 or PC12 cells) were seeded into the main channel at a density of 2×10^7 cells/ml through inlet 3. The cells in the channel were left undisturbed for 2 h at 37 ºC in a CO_2 incubator, which allowed them to settle within the microwells. The culture medium was then perfused into the main channel from inlets 1 and 2 at 0.2 µl/min, and untrapped cells washed away as a result of the cell-repelling property of the albumin-coated surface. After 3 days of perfusion culture, the remaining cell-repellent surface was converted to a cell-adhesive surface by introduction of 10 µg/ml PEI solution from inlet 4 for 5 minutes thereby facilitating the adhesion of secondary cells. Immediately after the treatment, 3T3 fibroblasts were seeded at a density of 3×10^7 cells/ml through inlet 3 to fabricate the coculture of 3D spheroids with two-dimensionally extended fibroblasts.

To validate the patterns in the coculture, the primary cells (HepG2 or PC12) and the secondary cells (fibroblasts) were fluorescently visualized with a cytoplasmic tracer (CFSE-green, Invitrogen) and a membrane-labeling dye (PHK26-red, Invitrogen) before seeding, respectively. Fluorescent micrographs were taken with a IX71

microscope (Olympus, Japan). To identify viable cells in spheroids, dual fluorescent staining was carried out using fluorescein diacetate and ethidium bromide. Fluorescein diacetate stains viable cell cytoplasm green, whereas ethidium bromide stains dead cell nuclei red.

Combinations with the gradient or focusing flow generators

The branched channel and 2 side channels that were placed above the main channel were used as gradient and focusing flow generators, respectively (18, 19). To investigate whether a concentration gradient and focusing flow can be generated even in the presence of 3D spheroids, the streams in the main channel were visualized using a fluorescent dye. After 3 days of perfusion culture, 70 mM fluorescein and PBS solutions were injected through inlets 1 and 2, respectively, both at a flow rate of 0.5 μl/min. A fluorescent image of the concentration gradient was taken 6 mm downstream from the entrance of the main channel and the profile was compared with the profile estimated from numerical calculations (19, 20). A focusing flow was also evaluated by introducing the fluorescein solution from inlet 1 and PBS solutions from inlet 5 and obtaining fluorescent images. The flow rates of the fluorescein and PBS solutions were set as 0.2 μl/min and 0.8 μl/min, respectively.

RESULTS AND DISCUSSION

Spheroid microarray and cocultures in a microchannel

Hepatocyte spheroid culture is a well-known culture method in which cells are induced to form spherical aggregates. Within these cultures hepatocytes better maintain their function and have been shown to have a cuboidal shape, and express intercellular adhesion molecules that are required for cellular communication (21, 22). Furthermore, hepatocyte spheroids exhibit liver-specific functions such as albumin synthesis and drug metabolism for extended periods of time (23). In this study, a microarray of hepatocyte spheroids was prepared inside a microchannel.

We have previously reported that a surface coated with cross-linked albumin is nonadhesive to cells and can be switched to favor adhesion of cells by electrostatic adsorption of a cationic polymer such as PEI (24, 25). Here we used this unique property to fabricate spheroid arrays and subsequent cocultures within microfluidic channels (Fig. 1B). To do this, we selectively coated the outside of the microwells with cross-linked albumin. The topography of the microwells created conditions in which air is remained in the wells to form a bubble when an aqueous fluid is introduced into a hydrophobic PDMS channel. This property was used to prevent the adsorption of the

albumin on the inner surfaces of the microwells. After washing the microchannels to remove non-adsorbed albumin, HepG2 cells were flowed into the channel and induced to seed in the microchannels. The cells that did not dock in the microwells were washed away, whereas the cells in the microwells remained in the wells due to shear protection from the flowing fluid in the channels. After 3 days in culture the 3D spheroids grew in size to generate fully formed spherical aggregates (Fig. 2A−D). The non-adhesive nature of the cross-linked albumin coated surface prevented the invasion of cells into the surface between the microwells for the 3 day study. The electrostatic adsorption of PEI to the negatively charged cross-linked albumin converted the surface to cell adhesive, which facilitated the subsequent deposition of secondary 3T3 cells and generated a coculture of hepatocyte spheroids with a fibroblast monolayer (Fig. 2E and F). Some cell types such as hepatocytes, pancreatic cells, neural stem cells, and embryonic stem cells require benefit from being cultured in 3D to maintain their metabolism, growth activity, and induce differentiation. These culture modes are called “spheroid,” “neurosphere,” or “embryoid body”. We expect that our system, which represents a combination of 3D and 2D cultures, will be useful for enhancing the efficacy of coculture.

Another factor that affects the cell fate is the degree of homotypic and

heterotypic cell-cell interactions. In our system, the size and shape of micropatterns can be changed relatively easily by changing the mask patterns. This can be used to control of the degree of cell-cell interactions through spatial localization of 2 cell types relative to each other. To demonstrate the utility of the proposed system for different size and shape well geometries, square and landscape-oriented rectangular microwells were fabricated and seeded with HepG2 cells. HepG2 cells formed 3D aggregates corresponding to the shapes of the microwells after 3 days of culture (Fig. 3A and B) and could be cocultured with fibroblasts (Fig. 3C and D). Furthermore, to show the utility of the approach with different cell types, PC12 cells were also docked in the rectangular microwells and induced to form patterned coculture with fibroblasts (Fig. 3E−H). Since neuron cells *in vivo* project axons toward their appropriate targets through crowded cell layers in response to a concentration gradient of axon guidance molecules during development and regeneration, the experimental protocol shown here could be combined with the concentration generator described below to provide a potentially beneficial tool for investigating the phenomenon of nerve regeneration and provide a platform for discovery of drugs which promote this process. One limitation of this approach is that the dimensions of microwells are required to be small enough to trap air bubbles. When microwells with 300 μm in diameter were used, some microwells were

coated with albumin from which cells easily come out during perfusion culture.

Viability of hepatocyte spheroids in microchannels

Viability of HepG2 spheroids were examined in the microfluidic channels after 3 days of perfusion. The live/dead fluorescent staining shows that most of the cells were viable (green) and only a few cells died (red) (Fig. 4A and B). Estimated from the medium flow (0.4 μl/min) the cells outside the microwells were subjected to a shear stress of <1 dyne/cm^2, which is significantly low relative to physiological shear stress in the venules (1–5 dyn/cm^2) (26). In spheroid culture, the depletion of oxygen causes necrosis in the core of a spheroid. The limitation distance in which cells can survive has been investigated in calculations and experiments. This distance is 80–100 μm from the surface of a cell aggregate (27-29). In the microwells, although the upper side of the aggregate faces the flowing culture medium, the cells in the bottom portion would have an unfavorable oxygen supply. On the other hand, the base substrate used to form the microwells consists of PDMS in contrast to typical microfluidic systems that utilized a rigid glass substrate as a base layer. Oxygen is 9-fold more soluble in PDMS than in culture medium. Furthermore, the oxygen diffusion coefficient of PDMS is twice as high as that of the culture medium (30-32). Thus, with regard to oxygen supply, our

system may provide a preferable environment for spheroid culture.

Concentration gradient and focusing flow formations

We have previously demonstrated that the migration of fibroblasts in a microfluidic channel can be studied in response to exposure to a concentration gradient (19). In this study, however, there is a concern that the presence of 3D spheroids may disturb the flow stream and interfere with the formation of a stable concentration gradient. To examine this issue, fluorescein and PBS solutions were injected into the main channel through inlets 1 and 2 in the presence of HepG2 spheroids after 3 days of culture and fluorescent images were obtained for analysis (Fig. 5A). As it can be seen the gradient was formed in the main channel, but the image analysis revealed differences in the generated concentration profile at the 3 positions investigated (Fig. 5B). There are also large differences between the experimental and calculated profiles.

We also demonstrated that hydrodynamic focusing of multiple streams can be used to spatially organize multiple streams on the array of cells. Interestingly, the focusing flow was also affected by the presence of HepG2 spheroids (Fig. 5C). Despite the introduction of the PBS solution through the precisely symmetric microchannel from a single inlet (inlet 5) the center of the focusing flow was shifted on 1 side by ~80

μm (Fig. 5D). In addition, the stream line appeared to be bent and stagnant. The width of the fluorescein band is ~200 μm in the 1.0 mm-wide channel, which is compatible with the flow ratio of 1:4 used in this study. On the basis of these results, a preliminary determination of a profile will be required for each experiment in order for precise applications of the gradient or focusing flow generators to be used for the 3D spheroid culture.

In conclusion, an approach for engineering a platform of spheroid microarrays inside a microchannel was demonstrated. This approach is based on features of cross-linked albumin and microwells fabricated on the bottom of a microchannel. Cells which were docked within the microwells formed 3D spheroids which remained stable in the microchannels. The albumin was complexed with PEI, which allowed for subsequent adhesion of secondary cells. This spatially controlled spheroid coculture system has potential as a useful tool for fabricating biomimetic cellular microenvironments.

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FIGURE LEGENDS

FIG. 1. Microfluidic device integrated with spheroid microarrays. (A) Configurations of microchannels, inlets and an outlet fabricated by stacking 3 layers of PDMS. The middle PDMS layer consisted of a main channel for the cell culture, a branched channel for generating a concentration gradient, 2 side channels for cell seeding and for generating a focusing flow in the main channel. The microchannels in the upper layer are for removal of air bubbles. In the lower layer, an array of microwells is formed at the position corresponding to the main channel in the completed device. The inset is a scanning electron micrograph of the microwells. The microwells were 100 μm in diameter and 70 μm in depth. (B) Steps for preparation of 3D hepatocyte spheroid arrays and cocultures with another cell type.

FIG. 2. Microarray of HepG2 spheroids on the bottom of the main channel and coculture with 3T3 fibroblasts. (A−D) Microarrayed HepG2 spheroids. Phase contrast (A−B) and fluorescent (C−D) images at 3 days of culture. (E−F) Fluorescent images of coculture of HepG2 cells (green) with 3T3 fibroblasts (red) at 1 day of culture after the secondary cell seeding. All scale bars represent 100 μm.

FIG. 3. Micropatterned spheroids in microwells with different shapes. (A−B) HepG2 cells were captured and induced to aggregate in square microwells for 3 days. The microwells were 150 μm on a side and 70 μm in depth. (C−D) Phase contrast (C) and fluorescent (D) images of the coculture of HepG2 cells (green) and 3T3 fibroblasts (red) at 2 days of culture after the secondary cell seeding. (E−F) PC12 cells were captured and induced to form an aggregate in a row of the rectangular microwells for 3 days. The microwells were 300 μm in length, 60 μm in width, and 20 μm in depth. (G−H) Phase contrast (G) and fluorescent (H) images of the coculture of PC12 spheroids and 3T3 fibroblasts (red) at 1 day of culture after the secondary cell seeding. All scale bars represent 100 μm.

FIG. 4. Live/dead fluorescent staining of spheroids in microfluidic culutre. (A) Majority of the HepG2 cells in the spheroids were viable (green, fluorescein diacetate) with only a few dead cells (red, ethidium bromide) at 3 days of culture in the microwells. (B) Magnified view of a microwell. Scale bars represent 50 μm.

FIG. 5. Influence of an array of spheroids on the concentration profiles formed with the gradient and focusing flow generator. (A) A concentration gradient of fluorescein

formed with the gradient generator at 6 mm downstream from the entrance of the main channel. The gradient was visualized by introducing fluorescein and PBS solutions from inlets 1 and 2, respectively. (B) Profiles of the concentration estimated by numerical calculations (red, dashed-two dotted line) and image analysis at the 3 positions in the image shown in (A). The labeled numbers are corresponding to the positions. (C) A focusing flow generated by introducing fluorescein solution from inlet 1 and PBS solution from inlet 5. (D) Profiles of the concentration estimated by image analysis at the 3 positions in the image shown in (C). The labeled numbers are corresponding to the positions. Scale bars represent 100 μm.