Micropatterned co-culture of hepatocyte spheroids layered on non-parenchymal cells to understand heterotypic cellular interactions

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Abstract
Microfabrication and micropatterning techniques in tissue engineering offer great potential for creating and controlling cellular microenvironments including cell–matrix interactions, soluble stimuli and cell–cell interactions. Here, we present a novel approach to generate layered patterning of hepatocyte spheroids on micropatterned non-parenchymal feeder cells using microfabricated poly(ethylene glycol) (PEG) hydrogels. Micropatterned PEG-hydrogel-treated substrates with two-dimensional arrays of gelatin circular domains (φ = 100 µm) were prepared by photolithographic method. Only on the critical structure of PEG hydrogel with perfect protein rejection, hepatocytes were co-cultured with non-parenchymal cells to be led to enhanced hepatocyte functions. Then, we investigated the mechanism of the functional enhancement in co-culture with respect to the contributions of soluble factors and direct cell–cell interactions. In particular, to elucidate the influence of soluble factors on hepatocyte function, hepatocyte spheroids underlaid with fibroblasts (NIH/3T3 mouse fibroblasts) or endothelial cells (BAECs: bovine aortic endothelial cells) were compared with physically separated co-culture of hepatocyte monospheroids with NIH3T3 or BAEC using trans-well culture systems. Our results suggested that direct heterotypic cell-to-cell contact and soluble factors, both of these between hepatocytes and fibroblasts, significantly enhanced hepatocyte functions. In contrast, direct heterotypic cell-to-cell contact between hepatocytes and endothelial cells only contributed to enhance hepatocyte functions. This patterning technique can be a useful experimental tool for applications in basic science, drug screening and tissue engineering, as well as in the design of artificial liver devices.

Keywords: micropatterning, spheroid, co-culture, cell–cell interactions, primary hepatocyte

1. Introduction
Liver tissue engineering, drug metabolism studies and many other applications would benefit from in vitro culture of hepatocytes that exhibit high levels of liver-specific functions over an extended period of time [1–4]. However, freshly isolated hepatocytes are known to readily lose their characteristic morphology, polarity and many differentiated functions during in vitro culture. The reconstruction of functional hepatic tissue is dependent on the ability to control factors that influence the cell environment, including cell–matrix interactions, soluble stimuli and cell–cell...
interactions [5–9]. Cell–cell interactions play a critical role in tissue morphogenesis and organ development. In vivo, the liver is a structurally and functionally heterocellular construct, composed of primary hepatocytes, endothelial cells, Kupffer cells, stellate cells and fibroblasts [5]. In an effort to reconstruct liver tissue in vitro for therapeutic applications, several studies have demonstrated that co-culturing primary hepatocytes with non-parenchymal cell types such as fibroblasts or endothelial cells maintains hepatocyte viability and function, whereas hepatocytes cultured alone rapidly lose their function [10–13]. However, these conventional co-culture methods (i.e. mixing the two cell types at random) are not capable of controlling the cellular interactions and spatial signaling that occur in the in vivo hepatic microenvironment.

It is necessary to establish a culture environment that controls the interactions between heterotypic cell populations. Micropatterning is such a technique and was used to control cell–cell and cell–surface interactions. [1, 2, 14–19]. Various techniques have been used to create micropatterned cells including photolithography, poly(dimethylsiloxane) elastomeric stencils and direct cell printing. Two-dimensional (2D) spheroid microarrays using microfabrication techniques have been utilized to mimic in vivo-like tissue structure [20–22]. These cell micropatterning techniques allow co-cultures to be created in which the cell density and the total length of contact between the two cell populations (‘heterotypic interface’) can be controlled. Using micropatterned islands of hepatocytes with surrounding 3T3-J2 fibroblasts, the previous studies demonstrated that the primary hepatocyte phenotype in vitro was stabilized as the length of the heterotypic interface increased [23, 24].

Based on these studies, we have previously reported that co-cultivation of hepatocyte spheroids layered on micropatterned endothelial feeder cells was essential to stabilize hepatocyte functions due to significant increase of heterotypic interface [20, 25]. The hepatocyte functions in such three-dimensional (3D) co-culture configurations were significantly higher than in the 2D co-culture. This suggests that 3D interactions are important for the maintenance of hepatocyte functions in vitro, which could contribute to other soluble factors or cell–cell interactions. Despite extensive work in this area, there has been controversy with respect to whether the heterotypic cell–cell interactions or soluble factors are primarily responsible for the enhanced hepatocyte functions observed in co-culture. To better understand these co-culture mechanisms, this study has used layered, patterned hepatocyte spheroids on micropatterned non-parenchymal feeder layers that are sensitive to environmental variations [26]. In particular, to elucidate the influence of soluble factors on hepatocyte function, hepatocyte spheroids underlaid with fibroblasts (NIH3T3) or endothelial cells (bovine aortic endothelial cells (BAECs)) as feeder cells were compared with physically separated co-culture of hepatocyte monospheroids with NIH3T3 or BAEC by a trans-well culture (with a membrane inserted between two cell types) system. The current study also compares the morphological and functional changes of hepatocyte spheroids that have been cultured with NIH3T3 or BAECs as non-parenchymal cells.

2. Experimental section

2.1. Materials

The following reagents were used as received: dehydrated dichloromethane, benzene and isopropyl ether from Sigma Chemical Company, St Louis, MO; bovine plasma fibronectin (FN) from Nitta Gelatin, Japan; rabbit anti-bovine FN polyclonal antibody from Biogenes, UK; rabbit anti-rat albumin antibody, peroxidase-conjugated sheep anti-rat albumin and fluorescein isothiocyanate (FITC)-conjugated goat antibody against rabbit immunoglobulin G (IgG) from Cappel, Aurora, OH; Dulbecco’s modified Eagle’s medium (DMEM) and Williams’ medium E from Invitrogen Corp., Carlsbad, CA, USA; fetal bovine serum (FBS) from PAA Laboratories, Exton, PA. Water used in this study was purified by a Milli-Q system (Nihon Millipore Co., Tokyo, Japan) to have a specific conductivity of less than 0.1 μS cm⁻¹.

2.2. Synthesis of branched poly(ethylene glycol) derivative (4arm20 K)

Twelve grams (93.6 mmol) of 4-azide-benzoic acid was dissolved in 40 ml of thionyl chloride, and the mixture was heated under reflux for 1.5 h. The reaction was concentrated under reduced pressure, and a small amount of hexane was added thereto. The mixture was concentrated again under reduced pressure, and dried under vacuum, as a result of which 9.3 g (51.2 mmol, yield 70%) of 4-azide-benzoic acid chloride, which is a target product, was obtained as a white solid. Proton nuclear magnetic resonance (1H-NMR; CDCl3) results: δ 8.11–8.15 (2H, m), 7.11–7.16 (2H, m).

Next, a solution of a branched poly(ethylene glycol) (PEG) (2 g, a pentaerythritol derivative having four PEG groups, n = 113) in dehydrated dichloromethane was dropwise mixed with a solution of the 4-azide-benzoic acid chloride, and was stirred at room temperature for 18 h. The reaction mixture was concentrated under reduced pressure, and suspended by adding benzene. The suspension was filtered to remove salt, and thereafter concentrated again under reduced pressure. The processes of dissolving the crude product in a small amount of benzene, dropwise adding the resultant solution to isopropyl ether cooled at 0 °C, and recovering the obtained precipitate by filtration, were repeated three times, and the obtained white solid was dried under reduced pressure. This procedure yielded 1.74 g (yield 85%) of a desired branched PEG derivative (4arm20 K). The replacement of the terminal hydroxyl groups by polymerizable substituents was evaluated as 90% from an integral ratio of 1H-NMR signals.

2.3. Preparation of 4arm20 K-coated micropatterned surfaces for cell culture

Micropatterned cell culture plate was fabricated according to the modified procedure of photolithographic methods [27] (figure 1). Briefly, poly-L-lysine-covered cover glass (Matsunami Glass Ind., Ltd; circular type having a diameter of 21 mm) was immersed in 0.15% (w/w)
Photomask
UV
Cover glass
PLL
Gelatin
Photoresist
coating
Photolithography

Figure 1. Schematic diagram showing sequence of steps in micropatterning. Poly-l-lysine-coated cover glass was further coated with gelatin. Then photosensitive branched PEG derivative (4arm20 K) was spin-coated on the gelatin-coated glass plate (500 rpm × 5 s + 3000 rpm × 20 s + 6000 rpm × 1 s). After natural drying, the film was brought into close contact with a photomask (on which a number of circular patterns, each having a diameter of 100 µm, are arranged), and irradiated to UV light for 40–120 s. After a development step by water, the film was dried, yielding a substrate with a microfabricated hydrophilic crosslinked material on its surface.

gelatin solution for 2 h. Then 110 µl of the photosensitive branched PEG derivative (4arm20 K) dissolved in toluene (1%) was dropped on the gelatin-coated glass plate, and a thin film was formed using spin coating (500 rpm × 5 s + 3000 rpm × 20 s + 6000 rpm × 1 s). After natural drying under ambient conditions, the film was brought into close contact with a photomask made of quartz plate (on which a number of circular patterns, each having a diameter of 100 µm, are arranged), and irradiated to light using a high-pressure mercury lamp (400 W) for 40–120 s. Thereafter, the film was washed with deionized water as a development step. The film was dried at room temperature, yielding a substrate with a microfabricated hydrophilic crosslinked material on its surface. Patterned substrate surfaces were visualized by fluorescence microscopy; the circular gelatin domain was confirmed by the adsorption of FN from an aqueous solution, immunostained in situ with rabbit anti-bovine FN polyclonal antibody, followed by a FITC-conjugated goat antibody against rabbit IgG.

2.4. Atomic force microscopy (AFM) characterization and analysis

AFM images were taken on an SPI3800 and S-image with Nano-Navi Station (Seiko Instruments Inc., Tokyo, Japan) in air and in water using the fluid cells that allowed imaging of the liquid-flooded samples. Topography measurements were conducted using V-shaped silicon nitride cantilevers bearing an integrated standard profile tip (nominal spring constant $K = 0.06 \text{ Nm}^{-1}$, DI-Veeco). The contact mode imaging utilized an applied load and scan rate limited to about 1 nN and 3 Hz, respectively, to minimize compression and lateral damage to polymer gels.

2.5. Experimental design

The trans-well co-culture experiments were performed to distinguish whether heterotypic direct cell-to-cell contact or soluble factors resulted in most improved hepatocyte functions. Co-culture of hepatocyte heterospheroids, physically separated co-culture of monospheroids and monolayer or monospheroids as a control were cultured as shown in figure 2.

2.5.1. Co-culture of hepatocyte heterospheroids underlaid with non-parenchymal cells (figure 2(a)). NIH/3T3 mouse fibroblasts (Health Science Research Resources Bank, Osaka, Japan) at passage 15 and BAECs (Japanese Collection of
Research Bioresources cell bank) at passage 13 were cultured with DMEM that was supplemented with 10% FBS, penicillin (100 units per ml) and streptomycin (100 µg ml⁻¹) at 37 °C in a humidified atmosphere with 5% CO₂. Rat primary hepatocytes were obtained from 6-week-old male Wister rats, as previously described, by using collagenase for cell dissociation [28]. The primary culture was plated in Williams’ medium E, supplemented with 10% FBS, penicillin (100 unit per ml) and streptomycin (100 µg ml⁻¹), and incubated at 37 °C in a humidified atmosphere with 5% CO₂ [28].

NIH/3T3 fibroblast cells or BAECs were seeded onto patterned dishes at a cell density of 3 × 10⁵ in the top insert. After 24 h of culture at 37 °C, unattached cells were washed away twice with the culture medium, then rat primary hepatocytes were seeded into these dishes at the same cell density and co-cultured at 37 °C with a cocktail medium (Williams’ E : DMEM = 1 : 1) in a humidified atmosphere with 5% CO₂ (Pattern I co-culture, Pattern II co-culture, respectively). Cell morphology was monitored under a phase-difference microscope (Axio Observer.D1, Carl Zeiss, Germany).

Physically separated co-culture of hepatocyte monospheroids with non-parenchymal cells by a trans-well culture system (figure 2(b)). Because controlling the organization of hepatocyte aggregates enhances their function even without the addition of non-parenchymal cells, the trans-well system used here was modified as illustrated in figure 2(b). As shown, the poly-l-lysine (PLL)-coated glass plate covered with a thin layer of gelatin and seeded with the non-parenchymal cells at a cell density of 3 × 10⁵ (e.g. NIH/3T3 fibroblast cells or BAECs) is placed in the bottom compartment of the trans-well system, and the glass plate with micropatterned hepatocyte monospheroids is placed in the top insert (Pattern III co-culture, Pattern IV co-culture, respectively).

2.5.2. Monolayer and monospheroid (figure 2(c)). For hepatocytes cultured as monolayer, rat primary hepatocytes were seeded onto the gelatin-coated glass plates at a cell density of 3 × 10⁵ and cultured at 37 °C with Williams’ medium E in a humidified atmosphere with 5% CO₂. For hepatocytes cultured as monospheroids, rat primary hepatocytes were seeded onto the micropatterned dishes at the cell density of 3 × 10⁵ and cultured at 37 °C with Williams’ medium E in a humidified atmosphere with 5% CO₂.

2.6. Cell viability and attachment

In order to evaluate cell viability and attachment in 2D monolayer and 3D spheroid cultures, 1 ml 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (500 µg ml⁻¹) was added to each well, and the incubation continued for 4 h. Finally, 1 ml of solubilization solution (isopropanol) was added. After 24 h of incubation, the absorbance of the resulting solution was measured at 570 nm using a 96-well microplate reader (Varioskan Flash, Thermo Fischer Scientific K. K., Yokohama, Japan). In all cases, test substrates were also added to wells containing medium alone without cells to determine possible interference with the colorimetric MTT assay. Transition of cell densities with time was calculated using standard curve of each cell by MTT. Data are shown as the mean ± standard error of the mean of three separate experiments, each performed in duplicate.

3. Results and discussion

Patterning is the ability to structure a material in two or three dimensions at various length scales. Patterning at length scales of microns further affords the opportunity to control interactions with specific proteins and individual cells [29]. In this study, base substrates followed by the micropatterning of PEG hydrogel were obtained by a usual layer-by-layer method [30, 31]. Polyelectrolyte complexes are theoretically formed by the reaction of a polyelectrolyte with an oppositely charged polyelectrolyte in an aqueous solution. Owing to amine functional molecules, the cation-charged PLL can interact with an anionic polyelectrolyte, e.g. poly(acrylic acid), sodium alginate, pectin, etc to form polyelectrolyte complexes. We have developed a polyelectrolyte complex of PLL and gelatin via electrostatic interaction. The glass surface was coated with PLL and gelatin in this manner, employing polyelectrolyte formation. Note that the outermost layer of this coating is assumed to be gelatin, which contains cell adhesive molecules including the tripeptide arginine-glycine-aspartic acid (RGD) peptide motif. Gelatin layers formed stable films of 2 nm thickness, which were confirmed by ellipsometry measurements, even after rigorous washing [32]. After that, micropatterning of PEG-coated surfaces was employed for 3D cell culture. Micropatterned PEG-gel-treated substrates with 2D arrays of gelatin circular domains (ϕ = 100 µm) were prepared by photolithographic method. Films of functional PEG macromers having tetra-functional molecules at the chain end can be patterned by selective exposure to ultraviolet (UV) light through a photomask (figure 3(a)). Unexposed regions can subsequently be washed away using water
as a good solvent for PEG, leaving behind patterns of crosslinked PEG hydrogel. Observation of the substrate using a phase-difference optical microscope confirmed the formation of a fine micropattern with 100 mm diameter (figure 3(b)). The PEG-treated region on the patterned substrate repels protein, and consequently inhibits cell adhesion [33–36]. PEG is known as a non-toxic and biocompatible polymer, and has been approved by the US Food and Drug Administration for both oral and topical applications. When present on a surface, PEG decreases the adsorption of proteins and the adhesion of cells. Several theories have been proposed to explain PEG’s antifouling behavior, including its large excluded volume, osmotic repulsion, high molecular mobility, lack of protein binding sites and high hydrophilicity [37–40]. Surfaces PEGylated by self-assembled monolayers and ultrathin films must be defect-free in order to create a robust interface between the substrate and the environment. Defects can expose the underlying substrate. There have been reports that monolayers and ultrathin films can lose their thickness over time [41] or become less antifouling because of oxidation [42]. Despite the fact that protein adsorption can be reduced substantially even with defective PEGylated surfaces, such surfaces exhibit imperfect resistance to cell adhesion. Consequently, thicker films and bulk PEG have attracted increasing attention, since they can be rendered biocompatible and are sufficiently thick, so that defects and degradation are less of an issue for longer-term application. Since PEG is water soluble, forming stable thick-film coatings requires the formation of hydrogels that can swell extensively but not dissolve. Hydrogels are networks of macromolecules stabilized either by chemical or physical crosslinks. In this study, we used chemically crosslinked hydrogels. One common way to create a covalently crosslinked network uses end-functional macromers. A polymerization reaction can be initiated between endgroups on different molecules. When propagated through an entire assembly of polymer in solid form, these create a continuous crosslinked network. The 4arm20K macromers, used in this study, have molecular weight of 20000 Da and functional phenyl azide groups at each of the four ends. The result of a representative synthesis is described below. The replacement of the terminal hydroxyl groups by photosensitive and reactive substituents calculated from an integral ratio of $^1$H-NMR signals was over 90%. $^1$H-NMR (CDCl$_3$): δ: 8.05 (8H, d, $J = 8.6$ Hz), 7.07 (8H, d, $J = 8.6$ Hz), 4.46 (8H, t, $J = 4.9$ Hz), 3.89–3.39 (2145H, m). Our crosslinking used in this study proceeds only by UV irradiation without requiring a photoinitiator whose toxicity may be of issue in clinical applications. Instead, the phenyl azide group generates a highly reactive nitren radical through UV irradiation, which spontaneously reacts with amines and hydrocarbons to form a covalent bond. One of the defining characteristics of a hydrogel is the extent of the reaction which can be controlled by the dose of radiation. In non-ionic PEG hydrogel, the extent of reaction can be derived experimentally from the swell ratio. When dry at UV irradiation time of 40 s, the diameter of each domain exposing a base gelatin is ∼100 mm and the height is ∼23 nm (figure 4). The substrate is gelatin, which becomes exposed because unirradiated PEG remains soluble in water and is washed away after irradiation. When hydrated, the hydrogels swell, and their height increases to 389 nm. Radiation chemistry must also occur between the gel and the gelatin surface in order to prevent the gel from washing away. Since these gels swell predominantly in only one direction, the swell ratio can be approximated as the wet height divided by the dry height [43], which corresponds to about 17 for the gels.

In this way, the crosslink density and, hence, the degree of swelling of photolithographically patterned PEG hydrogels can be controlled by the UV irradiation dose, since the UV lights are responsible for creating the reactive sites on the PEG precursor molecules. The critical gel formation to prevent proteins and cells depends on the dose time of UV irradiation. At UV irradiation time of 40 s, the obtained, loosely crosslinked gels with high swell ratio significantly resist the adsorption of FN, which is the major protein for cell adhesion, as one would expect on the basis of the well-established antifouling properties characteristic of PEGylated surfaces and PEG gels. For the present cell patterning, the region of PEG hydrogel should completely inhibit adsorption of proteins and cells, and thus an irradiation time of 40 s was employed.

On the other hand, proteins are expected to adsorb from the serum-containing medium onto the circular domains, exposing the base gelatin coated on glass substrate.
Figure 4. The result of AFM measurements. (a) AFM topography image of micropatterned PEG gel surface in dry state treated by photolithographic method, showing a gelatin circular domain ($\phi = 100 \mu m$). Cross-sectional images of the micropatterned substrate (b) before hydration (in dry state) and (c) after hydration (in wet state).

Figure 5. Patterned 3D co-culture of hepatocyte spheroids and non-parenchymal cells. (a) Patterned culture of 3T3 fibroblasts on substrate for 1 day at 37 °C. Primary rat hepatocyte spheroids were then layered on micropatterned 3T3 fibroblasts and cultured for (b) 1 day and (c) 10 days. (d) Patterned culture of BAECs on substrate for 1 day at 37 °C. Primary rat hepatocyte spheroids were then layered on micropatterned BAECs and cultured for (e) 1 day and (f) 10 days.

As a demonstrative adsorption of protein, FN was estimated to adsorb on the micropatterned surface. Indeed, the circular gelatin domains, separated by PEGylated regions, substantially adsorb FN as detected by immunofluorescence microscopy after treating with a fluorescein (FITC)-conjugated anti-FN antibody (figure 3(c)).

To assess the hepatocellular characteristics of micropatterned hepatocyte spheroids, we evaluated morphologic, phenotypic and functional characteristics of hepatocyte spheroids in physically separated co-culture with non-parenchymal layers and when co-cultured on non-parenchymal feeder layers. In performing tissue culture experiments involving two or more cell types, cell morphology and viability are useful indicators of the influence of the heterotypic cell interactions. In this study, the morphology of the hepatocytes was monitored in time using phase contrast microscopy. Figure 5 illustrates the day 1 and day 10 phase difference micrographs for hepatocyte heterospheroids. In Pattern I and II co-cultures, 3T3 fibroblasts at passage 15 or BAECs at passage 13 were seeded onto the patterned surfaces with $\phi = 100 \mu m$ circular domains that were edge-to-edge spaced at $l = 100 \mu m$ intervals (figure 3(b)), and cultured at 37 °C for 24 h in a 10% FBS medium. Obviously, 3T3 fibroblasts or BAECs adhered only onto the circular domains, exposing a gelatin surface (figures 5(a) and (d)). Preferentially adsorbed
Figure 6. The single cultures as layer and spheroid. Hepatocytes were seeded on gelatin-coated glass plate and cultured for (a) 1 day and (b) 10 days. Hepatocytes directly seeded on micropatterned substrate without preadhered non-parenchymal cells and cultured for (c) 1 day and (d) 10 days.

Figure 7. Transition of cell densities with time was estimated using standard curve of each cell by MTT. Data are shown as the mean ± standard error of the mean of three separate experiments, each performed in duplicate. (a) Single culture. (b) Co-culture.

extracellular-matrix proteins, including FN, vitronectin and laminin on the gelatin circular domains, may promote the adhesion of anchorage-dependent 3T3 fibroblasts and BAECs. This is supported by the fact that the adhered cell pattern is consistent with FN adsorption results, as seen in figures 3(b) and (c).

Rat primary hepatocytes, suspended in a culture medium, were then applied to the patterned dishes with cultured 3T3 fibroblasts or BAECs selectively located in the circular domains. Interestingly, rat primary hepatocytes formed spheroids within 24 h only on the circular regions of the existing 3T3 fibroblasts or BAECs, generating a two-dimensionally arrayed structure of the hepatocyte heterospheroids (figures 5(b) and (e)). In contrast, on the same patterned surface without preadhered non-parenchymal cells, hepatocytes attached to and formed monospheroids as a single culture on the circular gelatin regions (figures 6(c) and (d)). For monolayer culture as a control, rat primary hepatocytes were seeded on gelatin-coated glass plate (figures 6(a) and (b)). The hepatocytes cultured as monolayer exhibited rapid loss of their attachments. In contrast, hepatocyte monospheroids still existed on the micropatterned substrate, although their attachment might have been weakened (figures 6 and 7(a)). On the other hand, heterospheroids
co-cultured with 3T3 fibroblasts or BAECs retained their characteristic phenotypic morphology with distinct nuclei and well-demarcated cell borders. These results demonstrate the significant role of non-parenchymal cells as a feeder layer for the formation of hepatocyte spheroids. As suggested by viable cell counting (figure 7(b)), the hepatocyte viability of co-cultured heterospheroids was maintained. It is interesting to note that the effect of a feeder layer on the hepatocyte spheroid formation was clearly different between two cell types. The number of hepatocytes assembled on 3T3 fibroblasts (pattern I co-culture) was significantly higher than those underlaid with BAECs (pattern II co-culture). This is presumably due to the preferable cell–cell contact for hepatocytes with 3T3 fibroblasts.

Next, we measured albumin secretion as a function of time by using a sandwich ELISA to investigate the liver-specific functions for the hepatocytes in each micropatterned condition. As shown in figure 8, the albumin secretion rates from day 1 to 10 for the Pattern I co-cultured hepatocytes were increased compared with the corresponding values for the Pattern II co-cultured hepatocyte. The results also demonstrate that continuous albumin secretion was observed in both the hepatocyte heterospheroids co-cultured with non-parenchymal cells. The single cultures as layer and spheroid rapidly lose their functional characteristics, although the secreted albumin from monospheroids gradually decreased. Although the benefit of co-culturing micropatterned hepatocyte heterospheroids with 3T3 fibroblasts or BAECs (Pattern I and II co-cultures) is now clear, it is not known whether the effects are due to soluble factors or direct cell-to-cell contact. This was evaluated by a physically separated co-culture with 3T3 fibroblasts or BAECs (Pattern III and IV co-cultures) using a modified trans-well system. Figure 2(b) illustrates the trans-well system. The non-parenchymal cells (e.g. NIH/3T3 fibroblasts or BAECs) were seeded on the gelatin-coated glass plate and placed in the bottom compartment of the trans-well system, while hepatocyte monospheroids cultured on the micropatterned dishes were placed in the top insert. As a result, the effect of physically separated cells on the hepatocyte spheroid formation was clearly different between two cell types (figure 9). As clearly demonstrated by figure 7, the number of hepatocytes assembled on circular gelatin domain in Pattern III co-culture was significantly higher than those in Pattern IV co-culture. Furthermore, in pattern III co-culture, hepatocytes retained their characteristic phenotypic morphology even after 10 days of culture (figure 9). The reason why cells in the wells preferred aggregation to adhesion onto gelatin may be explained by the work of adhesion. Generally, hierarchy of cell–cell and cell–matrix interactions is described by the work of adhesion between them, which is analogous to molecules in a liquid solution [44]. Cells that express high levels of cell–cell adhesion molecules will have a large work of cell–cell adhesion. In our previous study, hepatocytes forming spheroids strongly expressed cortical actin organization outlining the cells, while the actin cytoskeleton in monolayer cells show stress fiber formation, which is linked to the cell–matrix contact via a focal adhesion complex [20]. Accordingly, the fact that hepatocytes kept the aggregated state means that the work of cell–cell adhesion was larger than that of cell–matrix adhesion. The phase-difference image shows the round hemispherical portion of the aggregates on the micropatterned surface (figure 9). Therefore, physically separated co-culture of hepatocyte spheroids with non-parenchymal cells resulted in the secretion of certain soluble factors into the culture media, leading to the enhanced cell–cell contact closely related to hepatocyte’s specific functions. Their ability for 3T3 fibroblasts may be preferable to that for BAECs. Indeed, Tzanakakis et al [45] reported that an intact F-actin network is required for both efficient spheroid self-assembly and liver-specific functions, including albumin secretion and cytochrome P450 activity, by using cytochalasin D, an inhibitor of actin polymerization [46].

To determine whether co-culture enhances the hepatocyte functions through soluble factors secreted into the culture media, hepatic albumin secretion was evaluated as a function of time.
Physically separated patterned 3D co-culture of hepatocyte spheroids with non-parenchymal cells using trans-well culture system. Hepatocytes directly seeded on micropatterned substrate were cultured with physically separated NIH/3T3 fibroblasts for (a) 1 day and (b) 10 days, or BAECs for (c) 1 day and (d) 10 days.

of time (figure 8). Physically separated co-culture of the BAECs and hepatocytes using the Transwell insert (Pattern IV co-culture) had a negligible effect on hepatocyte albumin secretions, whereas heterospheroids co-cultured with BAECs exhibited the retained hepatocyte functionality. This suggests that direct heterotypic cell-to-cell contact is more beneficial to hepatocyte performance when hepatocytes are co-cultured with BAECs. In contrast, co-culture of 3T3 fibroblasts and hepatocytes using the Transwell insert (Pattern III co-culture) resulted in the suppression of loss in hepatocyte albumin secretion levels, although the effect is smaller compared with heterospheroids with 3T3 fibroblasts (Pattern I co-culture). This indicates that the exchange of soluble factors contributes significantly to favorable hepatocyte performance in hepatocyte-3T3 fibroblasts co-cultures. In other words, both direct cell–cell contacts and soluble factors are important for enhancing hepatocyte functions in hepatocyte co-culture with fibroblasts. The results shown in figures 8 and 9 thus imply that the mechanism, through which co-culturing micropatterned hepatocyte spheroids with non-parenchymal cell populations benefits hepatocyte performance, varies with cell type.

4. Conclusions

We described a micropatterning technique to increase the heterotypic cell interactions in 3D co-cultures of hepatocyte spheroids with endothelial cells or fibroblasts. Hepatocytes were patterned to form spheroids on micropatterned endothelial cell or fibroblast feeder layers using micropatterned PEG hydrogel. To elucidate the influence of soluble factors on hepatocyte function, the results of micropatterned hepatocytes layered on feeder layers were compared with those of the trans-well culture system. Our results suggested that both direct cell–cell contacts and soluble factors are important for enhancing hepatocyte functions in hepatocyte co-culture with fibroblasts. In contrast, direct heterotypic cell-to-cell contact between hepatocytes and endothelial cells only contributed to enhance hepatocyte functions.

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