FORMATION OF COMPLEX HEPATIC ORGANOIDS USING MICROFABRICATED ANISOTROPIC HYDROGEL FIBERS

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ABSTRACT

A highly unique cell cultivation platform is presented to form linear hepatic organoids consisting of primary rat hepatocytes and feeder cells (Swiss-3T3 cells). Sodium alginate solutions containing hepatocytes/3T3 cells were introduced into a microchannel and gelled, to continuously incorporate cells into hydrogel microfibers, where hepatocytes at the center were closely sandwiched by 3T3. The hydrogel fiber-based cultivation enabled the formation of rod-like organoids with a length of 200~1000 μm, mimicking the hepatic-cord structures found in the liver, while keeping a high viability (>80%). Also, several hepatic functions (albumin and urea synthesis) were significantly enhanced compared to the conventional plate culture and the single cultivation in the hydrogel fibers.

KEYWORDS: Hydrogel fiber, Hepatocyte, Cell cultivation, Co-culture

INTRODUCTION

Hepatocytes (liver parenchymal cells) are the highly functional cells often utilized in various research, clinical, and pharmaceutical applications, including the drug screening assays and the fabrication of bio-artificial livers. Hepatocytes are known to have the significantly high regeneration ability *in vivo*, but rapidly lose their functions when they are isolated from the liver and cultured *in vitro*. Researchers have therefore developed techniques to maintain the hepatic functions *in vitro*, including the patterned co-culture with feeder cells [1], spheroid formation [2], and the use of hollow fibers to form linear organoids [3].

Hepatocytes in the liver are arranged in cord-like structures known as the hepatic cord, which are surrounded by non-parencymal cells (ex. stellate and sinusoidal endothelial cells) (Figure 1). This structure allows each hepatocyte to be in close contact with the blood flow in the sinusoid, enabling the effective exchange of nutrition, oxygen, metabolic products, etc. It is anticipated that the microenvironments mimicking the *in vivo* liver structures would improve the hepatic functions, even when they are cultured *in vitro*. In this study, we utilized the previously developed co-flowing microfluidic system [4] to produce anisotropic alginate hydrogel fibers, in which hepatocytes are aligned in a row and feeder cells (Swiss 3T3 cells) are located on both sides (Figure 2 (a)). In the hydrogel fibers, aggregated hepatocytes are surrounded by 3T3 cells, forming three-dimensional and linear organoids that highly mimic the liver cord structures (Figure 2 (b)). In addition, to investigate the effect of the co-culture with 3T3 cells, several hepatic functions were analyzed and compared to the conventional plate culture and the single culture in the hydrogel fibers.

EXPERIMENTAL SECTION

PDMS microdevices were fabricated by using standard soft lithography and replica molding techniques. The microchannel width was 400 μm, while the depth was changed from 80 to 200 μm (Figure 2 (a)). Primary rat hepatocytes were isolated from 8~12-week-old male F344/N rats by the collagenase perfusion method, while Swiss 3T3 cells (mouse embryonic fibroblast cell line) were cultured in DMEM supplemented with FBS and harvested by trypsin treatment. Hepatocytes and 3T3 cells were individually suspended in an isotonic solution of sodium alginate. These suspensions of primary rat hepatocytes and 3T3 cells were continuously introduced into the microchannel from the center inlets at flow rates of 10-30 μL/min by using syringe pumps. Aqueous solutions of 10% (w/v) dextran, with and without containing the gelation agent, were respectively used as the gelation and buffer solutions. After the synthesis and recovery of the cell-containing alginate hydrogel fibers, cells were cultured in a cell-cultivation medium for up to 90 days. The concentrations of the produced albumin and urea in the medium were measured by ELISA and colorimetric assay, respectively, while real-time RT-PCR was

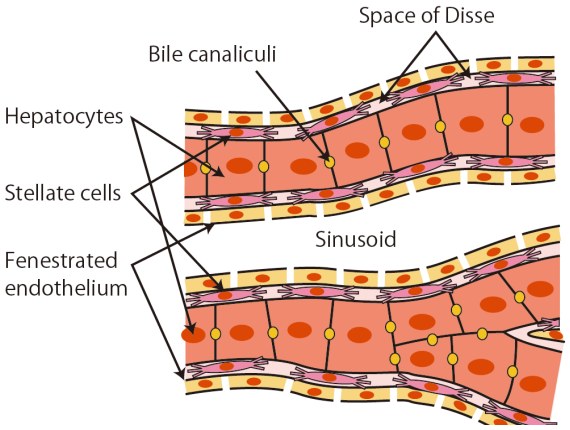

Figure 1: Schematic illustration showing the hepatic-cord structure of the liver. Hepatocytes form cord structures, which are surrounded by the layers of non-parenchymal cells like stellate cells and endothelial cells.

conducted to analyze the rat-specific mRNA expression in hepatocytes.

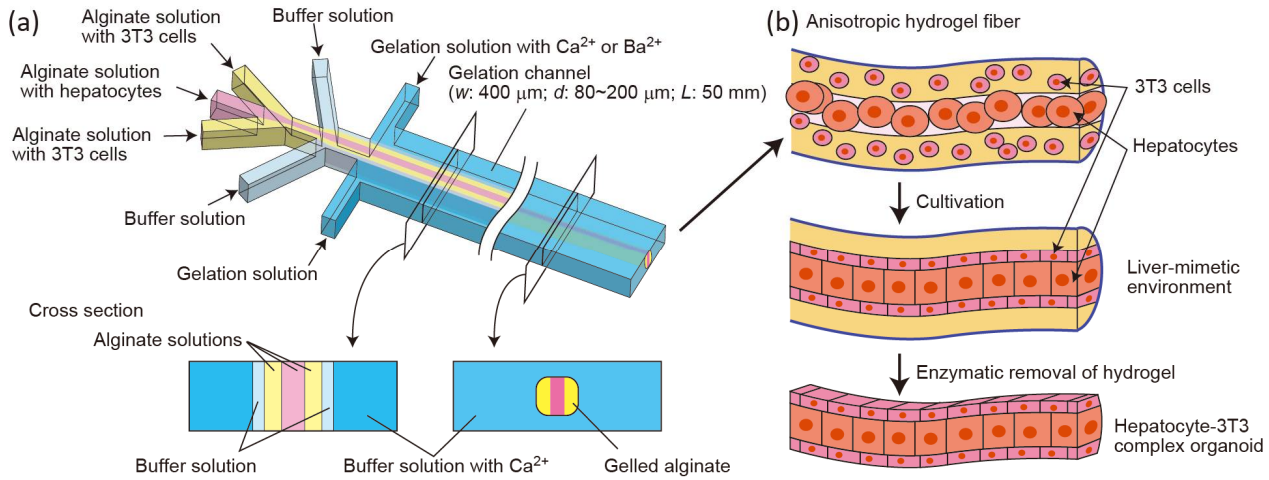

Figure 2: (a) Microfluidic system for preparing anisotropic hydrogel fibers incorporating hepatocytes and 3T3 cells. (b) Formation process of complex hepatic organoids mimicking the hepatic cord, by using the anisotropic fibers.

RESULTS AND DISCUSSION

Figure 3 shows the multiphase flows in the microchannel. By adjusting the flow rates of each solution, we successfully obtained the cell-incorporating alginate hydrogel microfibers, with a diameter of 50-70 μm (Figure 4 (a)). Hepatocytes (diameters of 20-30 μm) were aligned in one or two rows in the core (width of 30-50 μm), which were sandwiched by the shells (width of 10-20 μm) containing 3T3 cells. The production rate of the fiber was sufficiently high, 5-10 m/min, with the corresponding number of hepatocytes of 5-10 × 10^6 cells/min. After cultivation for several days, hepatocytes and 3T3 cells formed linear aggregates inside the fibers (Figure 4 (b)). The viabilities of hepatocytes just after preparing the hydrogel fibers and after 7 days of cultivation were kept high, ~90% and ~80%, respectively.

Then we recovered the formed organoids by enzymatically digesting the alginate fibers using alginate lyase. Figure 5 (a) and (b) shows the bright-field image of the formed organoids, with a length of 200-1000 μm and a width of 50-70 μm. The cell positions in the hydrogel fibers were investigated by immunohistological staining, as shown in Figure 5 (c) and (d). Hepatocytes were stained in green with anti-rat albumin antibody, while the nuclei of the hepatocytes and 3T3 cells were stained in blue with Hoechst. It was confirmed that the linear aggregates of hepatocytes were surrounded by a single layer of 3T3 cells after 7 days of cultivation, although the initial positions of 3T3 cells were only on both sides of the hepatocytes. These layered organoids highly mimic the hepatic cord structures found in the liver.

Finally, we examined several hepatic functions, and compared the results with the conventional 2D plate culture and the 3D fiber culture without employing 3T3. Figure 6 (a) shows the time-course albumin secretion in the medium, measured by ELISA. After ~1 week of cultivation, the albumin production was significantly enhanced for the co-culture in the hydrogel fiber, and this level was kept high up to ~60 days of cultivation *in vitro*. Similar tendencies were observed for the urea synthesis. Figure 6 (b) show the expression of ornithine transcarbamylase (OTC, associated with urea synthesis) genes analyzed by real-time RT-PCR. The expression of this gene was also enhanced in the co-culture system in the hydrogel fiber. These results clearly demonstrated that the microscale 3-D co-culture system, which highly mimics the *in vivo* sinusoidal structures, is a powerful means to maintain the hepatic functions *in vitro*.

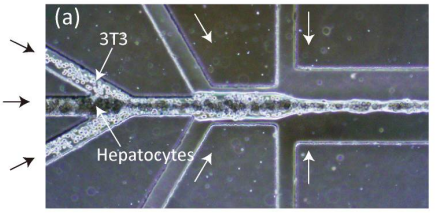

Figure 3: Formation of the anisotropic hydrogel fibers in the PDMS microchannel at (a) the confluence point and (b) 25 mm from the confluence.

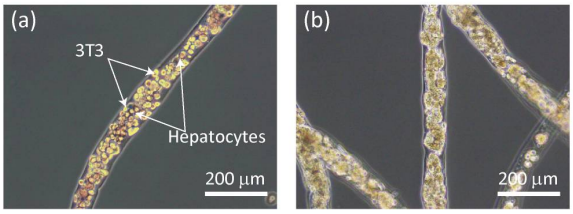

Figure 4: Micrographs showing the anisotropic fibers after (a) 0 and (b) 7 days of cultivation.

CONCLUSIONS

Sandwich-type anisotropic hydrogel fibers, having multiple regions of different cells in the cross section, were utilized to form complex organoids composed of primary hepatocytes and 3T3 cells. The presented technique is highly effective to maintain the hepatic functions *in vitro* for a relatively long period of time (~60 days), and is advantageous for conducting hepatocyte-based drug-screening applications and constructing the bio-artificial liver models.

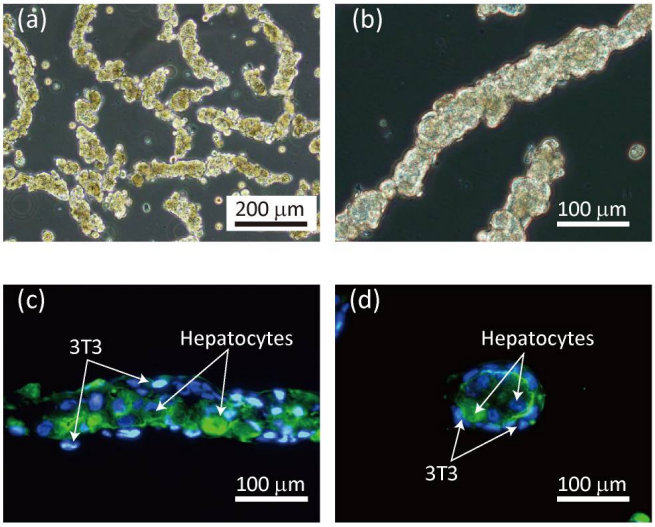

Figure 5: Hepatocyte-3T3 organoids obtained by enzymatically digesting the alginate hydrogel after 7 days of cultivation. (a,b) Optical micrographs of the organoids, and (c,d) fluorescence micrographs showing the horizontal and vertical cross-sections of the hepatic organoids. In (c, d), cell nuclei (both hepatocytes and 3T3) were stained in blue (with Hoechst) while hepatocytes were stained in green (with FITC-conjugated anti-rat albumin).

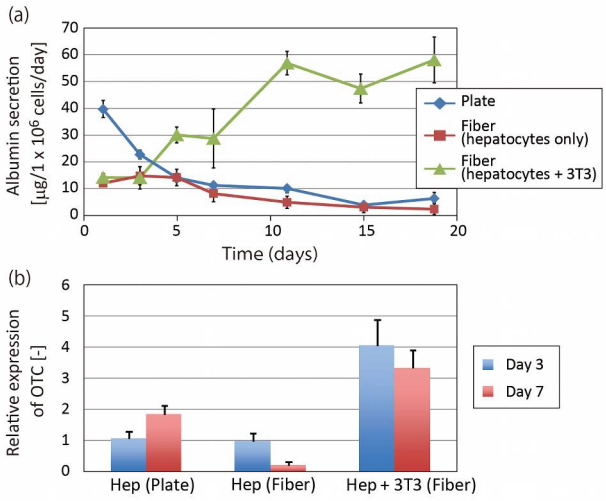

Figure 6: Evaluation of the hepatic functions in the co-culture in the anisotropic hydrogel fibers, in comparison to the 2D plate culture and the single culture in the fibers. (a) Time-course secretion of rat albumin analyzed by ELISA and (b) expression of ornithine transcarbamylase (OTC, associated with urea cycle) gene analyzed by real-time RT-PCR.

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