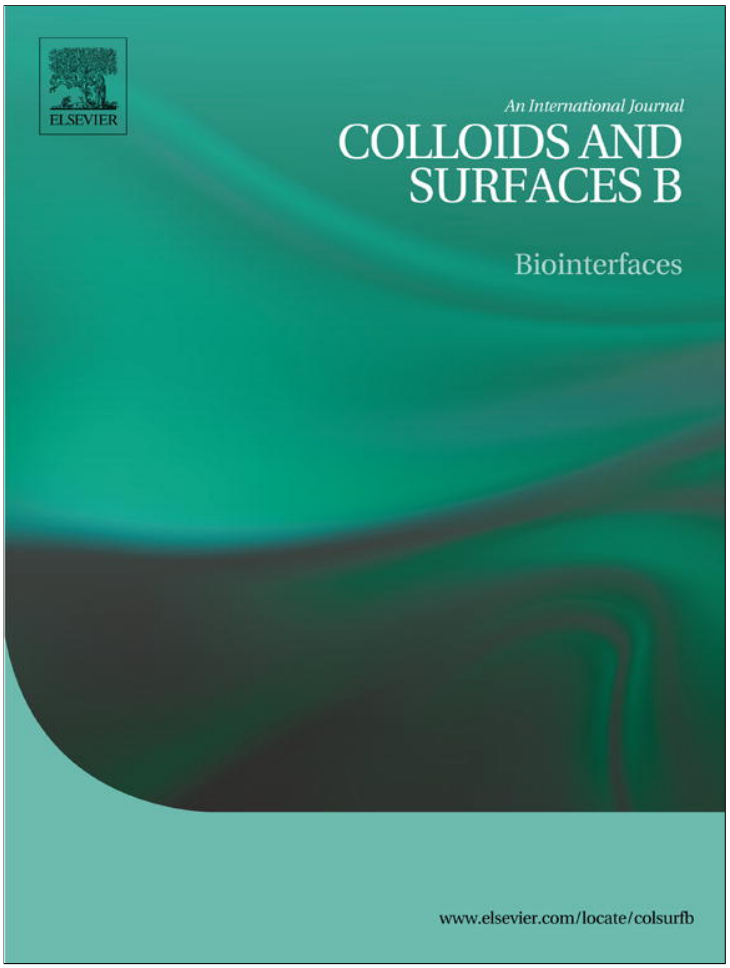

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Colloids and Surfaces B: Biointerfaces 97 (2012) 97–100

Contents lists available at SciVerse ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Long-term survival and functional maintenance of hepatocytes by using a microfabricated cell array

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ARTICLE INFO

Article history:
Received 23 March 2012
Accepted 15 April 2012
Available online xxx

Keywords:
Microfabricated cell array
Spheroid
Liver diseases
Cytochrome P450
Albumin secretion

ABSTRACT

We developed a microfabricated cell array of hepatocyte spheroids that showed long-term viability and retained the properties of the parent hepatocytes. Fresh hepatocytes harvested from 8-week-old Wistar rats were cocultured with feeder cells to rapidly form hepatocyte spheroids; these cells retained the spheroidal formation for 42 days. We also evaluated the cellular functions of the hepatocytes such as albumin secretion and metabolic activity of cytochrome P450 (CYP). In spheroids in which hepatocytes were cocultured with feeder cells, these cellular functions were retained even after 42 days. Therefore, this novel coculture will be very useful not only for research on the mechanism and treatment of liver diseases but also for early prediction of hepatocyte toxicity in the pre-clinical phase of drug development.

1. Introduction

Research on the mechanism and treatment of liver diseases has attracted considerable attention in recent years [1]. The number of patients with non-alcoholic steatohepatitis (NASH), which is caused by progression of non-alcoholic fatty liver disease (NAFLD), has been increasing. NASH progresses to liver cirrhosis, which in turn is followed by liver cancer. However, the detailed mechanism of this series of diseases is unclear, and treatments for these diseases have not yet been established. To analyze the mechanisms underlying liver diseases and to develop suitable drugs for these diseases, it is very important to establish an *in vitro* cell culture system that can reflect the actual properties of liver cells over an extended period of time, since such cultures would allow researchers to analyze long-term phenotypic and genotypic changes in hepatocytes.

Cell-culture systems that can exhibit liver-specific functions over an extended period of time are also important for early prediction of hepatocytotoxicity in the pre-clinical phase of drug development, because the U.S. Food and Drug Administration recommends the use of *in vitro* hepatocyte models to evaluate the cytochrome P450 (CYP) induction potential [2]. Monolayer cell-culture systems are conventionally used for this purpose; however, cells in these cultures often lose their original properties after prolonged incubation. The original properties of the cultured hepatocytes are better maintained in 2D or 3D cell culture systems (*e.g.*, spheroid cultures) than in monolayer cell culture systems. Microfabricated cell array systems are very useful for the high-throughput and high-performance screening. Otsuka et al. developed a 2D microarray of hepatocyte heterospheroids by cocultivating hepatocytes with endothelial cells on glass surface fabricated by plasma etch technique [3]. We previously constructed a PEG-gel micropatterned surface on a glass support by using the UV photolithography technique [4]. Hepatic spheroid of human hepatocyte cancer cell line and fetal mouse liver cell can be constructed easily and with high reproducibility on the constructed PEG-gel micropatterned glass surface [4,5].

In this report, we describe a novel cell chip system on a polystyrene-based plate surface that was microfabricated from a PEG block copolymer. The primary hepatocytes were harvested from the Wistar rats and were cocultured in a spheroidal form. This method yielded hepatocyte cultures that showed very long-term viability and excellent maintenance of hepatocyte functions such as albumin production and CYP activity.

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http://dx.doi.org/10.1016/j.colsurfb.2012.04.022

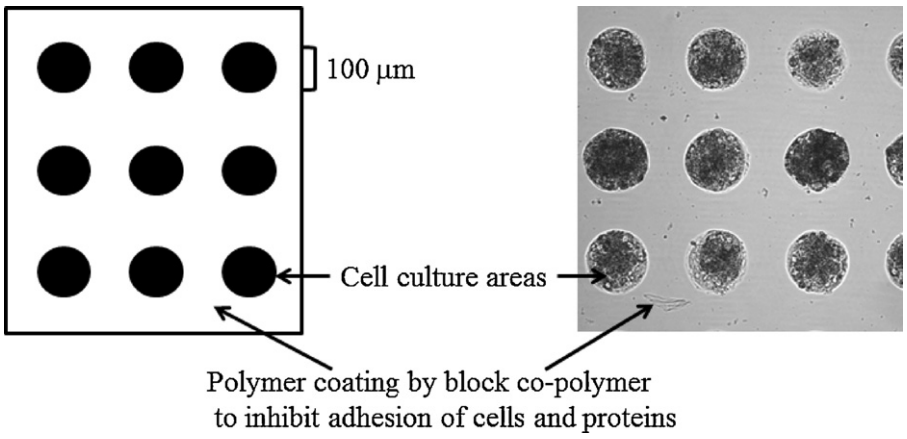

Fig. 1. Schematic representation of the Cell-able system and a phase contrast micrograph of microfabricated cell array of hepatocyte spheroids.

2. Materials and methods

2.1. Materials

Cell-able 96-well plates and culture medium for hepatocytes were purchased from Transparent Inc. (Chiba, Japan). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used without further purification.

2.2. Rat hepatocyte isolation and culture

Primary hepatocytes were harvested from 8-week-old Wistar rats by a 2-step *in situ* collagenase perfusion method. Cell viability determined by Trypan Blue exclusion assay was over 80%. Twenty-four hours before seeding of the hepatocytes, feeder cells (NIH/3T3) were seeded on the Cell-able 96-well plate at a density of 8000 cells per well. Freshly isolated rat hepatocytes were seeded in the hepatocyte culture medium at a density of 40,000 cells per well. The culture media were changed every other day.

2.3. Albumin secretion

The albumin concentration in the culture medium was analyzed by using the rat albumin ELISA quantitation kit (Bethyl Laboratories Inc.) according to the manufacturer's instructions. The medium was changed 24 h before the analysis.

2.4. Measurement of CYP activity

CYP activities were analyzed by assessing testosterone metabolism. Cultured cells were washed twice with the Williams' E medium containing 100 μM of testosterone. After washing, the cells were incubated for 4 h in Williams' E medium, which contained testosterone at final concentration of 100 μM. The medium was analyzed using liquid chromatography/mass spectrometry (LC/MS) to calculate the amounts of 6β-hydroxyl testosterone produced. A TSKgel ODS-120T column was used for the chromatographic analysis. The samples were eluted with an acetonitrile:H_2O (10:90 [at 0 min] to 60:40 [at 50 min]) solution at a flow rate of 0.4 mL/min. Blank medium served as the control to show the background absorbance values.

2.5. CYP induction assay

On day 4, the hepatocytes were treated with 2 mM PB to induce CYP activity. The culture media containing PB were replenished every 24 h for a total treatment time of 72 h. The metabolic activities of CYP were evaluated on the basis of testosterone metabolism by using the method described above.

3. Results and discussion

We used Cell-able system, a novel cell culturing system to construct rat-hepatocyte spheroids. This system consisted of a polystyrene-based plate, which was microfabricated from a block copolymer consisting of a polyethylene glycol (PEG) derivative containing an azide group as a photo cross-linker. Coating the surface of polystyrene with block co-polymer by photolithography inhibits the adhesion of proteins and cells. The non-coated area could accommodate spheroids with a diameter of 100 μm. Fig. 1 shows the construction of hepatocyte spheroids by using the Cell-able system.

Cultivation of spheroids was performed with or without feeder cells. For cocultivation with feeder cells, we incubated 3T3 fibroblasts for 24 h before layering fresh hepatocytes obtained from 8-week-old Wistar rats. After layering the hepatocytes, spheroid formation was confirmed by microscopy. Fig. 2 shows the images of cultured spheroids and monolayer cells. Spheroid formation in hepatocytes cultured with feeder cells was faster than that in hepatocytes cultured alone. Notably, when cultivated with feeder cells, the cultured hepatocytes retained their spheroidal form even after 42 days of incubation.

To investigate the cellular function of the hepatocyte spheroids, hepatic albumin secretion was evaluated as a function of time. The culture medium was changed 24 h before analysis, and albumin secretion was evaluated using a sandwich enzyme-linked immunosorbent assay (ELISA). The results are shown in Fig. 3. In spheroids created without feeder cells, the albumin secretion diminished after 2 weeks of culture; however, in hepatocytes cultivated with feeder cells, continuous secretion of hepatic albumin was observed for over 42 days after incubation.

To further investigate the cellular function of spheroids, the metabolic activity of the hepatocytes was evaluated. Testosterone was added to the spheroids at a final concentration of 100 μM for 4 h. The metabolic products in the medium were analyzed by high-performance liquid chromatography (HPLC). Several hydroxylated products of testosterone were detected in the HPLC analysis.

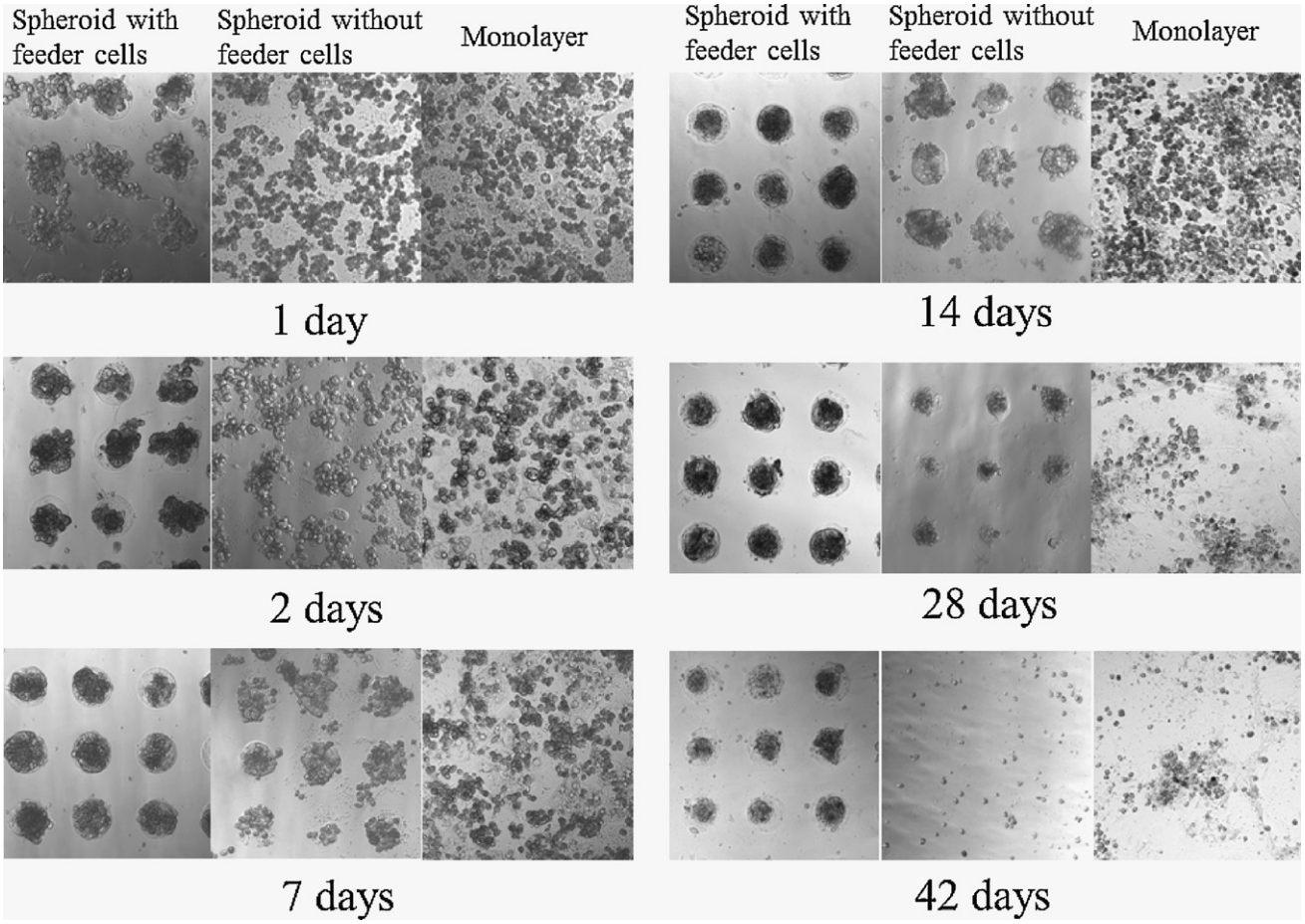

Fig. 2. Patterned cell array system of hepatocyte spheroids with or without feeder cells, which was prepared using the Cell-able system. A monolayer culture on the collagen-coated plate is also shown.

Among these, 6β-hydroxytestosterone was analyzed, because this compound is a major metabolite of testosterone by the action of CYP3A4 and CYP3A5 [6].

Fig. 4 shows the retention of metabolic activity in spheroids cocultivated with feeder cells. Compared to the diminished metabolic activities of monolayer cultures and spheroid cultures without feeder cells, the metabolic activity of spheroid cultures with feeder cells was retained even after 42 days of incubation. The potential induction of CYP activity was investigated because CYP induction is an important mechanism that reduces drug efficiency. After hepatic spheroids which were cocultured with feeder cells were exposure to phenobarbital (PB) for 72 h, which induces

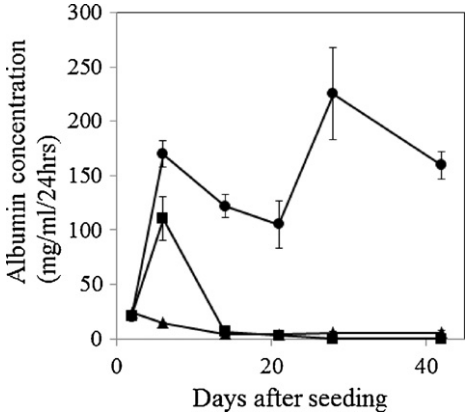

Fig. 3. Albumin secretion assay. Albumin secretion by hepatocyte spheroid cultures underlaid with or without feeder cells and by monolayer hepatocytes as a function of time. Closed circle, hepatocytes with feeder cells; closed square, hepatocytes without feeder cells; and closed triangle, monolayer hepatocytes.

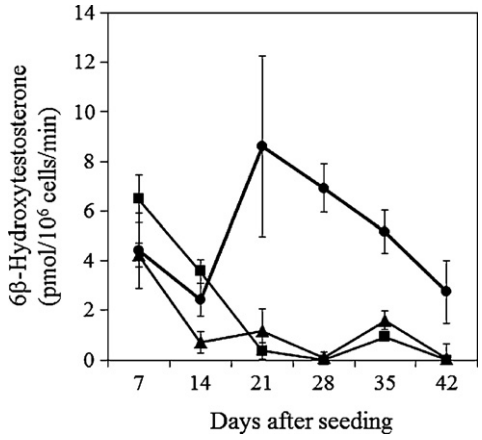

Fig. 4. Metabolic activity of hepatocytes. Cytochrome P450 (CYP) activities of spheroidal hepatocytes with or without feeder cells or hepatocytes in monolayer cultures evaluated by assessing the conversion of testosterone to 6β-hydroxytestosterone. Closed circle, hepatocytes with feeder cells; closed square, hepatocytes without feeder cells; and closed triangle, monolayer hepatocytes.

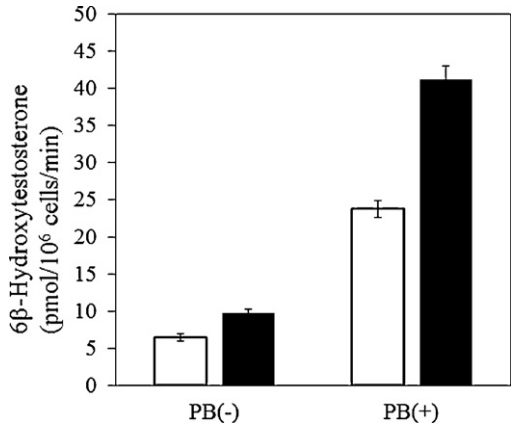

Fig. 5. CYP induction assay. Phenobarbital (PB) – an inducer of CYP – was incubated with hepatocytes with or without feeder cells. The activity of hepatocytes was evaluated by assessing the conversion of testosterone to 6β-hydroxytestosterone. Open bar, hepatocytes without feeder cells; closed bar, hepatocytes with feeder cells.

CYP activity, the metabolic activities of CYP were evaluated by assessing testosterone metabolism. The results are shown in Fig. 5. On addition of PB, a 4-fold increase was observed in the activity of CYP. These results confirmed that hepatocyte functions were maintained in the spheroid.

4. Conclusion

In this report, we described the formation and evaluation of rat primary hepatocyte spheroids on poly-styrene based plate. In spheroids that were cocultured with feeder cells, cellular functions such as hepatic albumin secretion and metabolic activity were retained for 42 days. It should be noted that continuous secretion of albumin and retention of function of the hepatocyte for over 42 days has been rarely reported. In recent times, the Cell-able system has found applications in the study of human hepatocyte cultures [7]. This system is a useful tool for proteomic and genetic analyses of liver diseases and for early prediction of the potential risks associated with new chemical entities during drug development.

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