<table>
<thead>
<tr>
<th>項目</th>
<th>内容</th>
</tr>
</thead>
<tbody>
<tr>
<td>項目</td>
<td>體細胞の研究分野では、肝細胞を模倣するためのモデルとして、ホジキン細胞を用いた研究を行ったもの。肝細胞の機能を保つために、デンキ・フィラメントを介して細胞の形態をコントロールする技術を用いた。</td>
</tr>
<tr>
<td>昇進</td>
<td>昇進</td>
</tr>
<tr>
<td>働き方</td>
<td>働き方</td>
</tr>
<tr>
<td>体制</td>
<td>体制</td>
</tr>
<tr>
<td>その他</td>
<td>その他</td>
</tr>
<tr>
<td>その他</td>
<td>その他</td>
</tr>
</tbody>
</table>

© 2014 長崎大学医学部
Rat hepatocyte spheroids formed on temperature-responsive PIPAAm polymer-grafted surface maintain long-term differentiated hepatocyte function

Tetsuo Tomonaga1, Akihiko Soyama1, Kosho Yamanouchi1, Mitsuhisa Takatsuki1, Tamotsu Kuroki1, Takehiko Koizumi1, Susumu Eguchi1

1 Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
2 Department of Histology and Cell Biology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Introduction

The development of effective culture methods is very important in regenerative medicine. This is because they may be able to contribute to the advancement of medicine. Since the liver plays an important and complex metabolic role, the development of a practicable culture method to maintain long-term hepatocyte function is highly desirable. However, hepatocytes lose their liver-specific functions rapidly on a regular collagen-coated dish (1). Developing an incubation system which can maintain the liver function would markedly contribute to regenerative medicine.

Recently, a newly developed culture dish was introduced coated with the temperature-responsive polymer poly(N-isopropylacrylamide) (PIPAAm) (2-9), whereby temperature fluctuations can mediate changes in the chemistry of the
polymer coating. At 37°C, PIPAAm is slightly hydrophobic, allowing cells to be cultured under normal conditions. A decrease in the temperature below 32°C, however, results in rapid hydration of the polymer, leading to the spontaneous detachment of cells as a uniform tissue sheet. As the temperature-responsive polymer is covalently immobilized onto the plate surface, PIPAAm remains bound to the dish even after cell detachment. Using this technology, tissue sheets composed of oral mucosal epithelial cells have been generated and are now clinically applied for corneal reconstruction, with this approach being noted for its simplicity and favorable clinical results (5). Moreover, it facilitates the growth of liver tissue ectopically using a hepatocyte sheet (10).

This culture system was originally developed for the study of cell sheets, but the impact of the amount of polymer on the morphology and function of cultured cells is not yet clear. In the current study, we investigated the possibility of developing a new culture system with PIPAAm, and the effect of changing the amount of polymer on the surface of the culture plate.

Materials and Methods

Animals

For all of the experiments performed in this study, male Sprague-Dawley rats (Charles River Laboratories, Yokohama, Japan), 10-14 weeks old, were used for harvesting hepatocytes. All animal experiments were performed in accordance with the institutional guidelines set forth by the Animal Care Committee of Nagasaki University. Rats were housed in cages within a temperature-controlled room with a 12-hour light/dark cycle, and given ad libitum access to food and water.

Hepatocyte isolation and purification

Hepatocytes were isolated and purified from the Sprague-Dawley rats using a modified 2-step collagenase perfusion method, as previously described (11). Isolated cells were filtered through a nylon mesh membrane and hepatocytes were then purified by slow-speed centrifugation at 50 G for 1 min performed 3 times. The viabilities of the isolated hepatocytes were determined with the trypsin blue exclusion test. Culture experiments were conducted only when the hepatocyte viability exceeded 90%.

Preparation of culture dishes

Culture dishes with different amounts of PIPAAm cova-

lently immobilized on their surfaces were prepared as previously described (12). In brief, varying amounts of N-isopropylacrylamide (IPAm) dissolved in 2-propanol were added and spread uniformly over 35-mm polystyrene tissue culture (PSTC) dishes (CORNING, Fisher Scientific, Rochester, NY, USA). The dishes were subjected to a 0.25-MGy electron beam to simultaneously graft and polymerize the PIPAAm. The presence of PIPAAm was confirmed by electron spectroscopy, and the homogeneity of the PIPAAm grafts on PSTC dishes was confirmed by field emission scanning electron microscopy and atomic force microscopy. The amount of grafted PIPAAm was determined by attenuated total reflection Fourier-transform infrared spectrophotometry. As a result, culture dishes with 5 different PIPAAm amounts grafted were prepared: (-), no-PIPAAm-grafted naïve PSTC dish; 1+, dish grafted with the same amount of PIPAAm as the commercially available PIPAAm dish (Up-Cell®, CellSeed, Inc., Tokyo, Japan); 2+ - 4+, dishes grafted with a higher amount of PIPAAm compared with the commercially available PIPAAm dish, as described in Figure 1.

Cellular attachment to the PIPAAm dishes was enhanced by coating the culture surfaces with rat tail collagen I (BD, Franklin Lakes, NJ, USA) at a dose of 600 µg/mL for 3 hours at 37°C in all groups. After this coating process, the dishes were washed twice with pre-warmed phosphate-buffered saline (PBS).

Hepatocyte culture

Isolated and purified rat hepatocytes were resuspended in William's E culture medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 10⁻⁷ M dexamethasone, 0.5 µg/ml insulin, 30 µg/ml L-proline, 10 ng/ml epidermal growth factor, 0.2 mM Asc-2P, 1% dimethyl sulfoxide (DMSO), 100 units/mL penicillin, and 100 mg/mL streptomycin. Cells were placed on 35-mm PIPAAm dishes at a rate of 1.1 x 10⁶ cells per dish and cultured for 14 days at a temperature of 37°C. On day 1, the culture medium was changed to remove unattached cells from the cell culture. The culture medium was changed every 48 hours thereafter.

Samples were obtained on days 1, 3, 7, and 14 of culture to assess morphologic changes and perform functional assays of cultured cells.

Morphological observation

The morphology of the cultured hepatocytes was assessed using a phase contrast microscope (ECLIPSE Ti, Nikon, Tokyo, Japan). During photographing, attention was
Measurement of albumin concentration

Albumin secretion into the culture medium was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using the Rat Albumin Quantitation Set (Bethyl Laboratories, Montgomery, TX, USA) according to the manufacturer's protocol. For each data point, 5 samples were analyzed per group.

Electron Microscopic examination

The cultured hepatic cells were fixed with 1.25% glutaraldehyde at 37°C, and 0.1 M phosphate buffer was added to the culture dish.

The sample was cut into ultrathin sections (thickness of 75-80 nm), and the sections were inspected with a transmission electron microscope (JEOL JEM1200EX).

Statistical analysis

All values calculated in this study are presented as the mean ± standard deviation. Statistical analysis was performed employing the Tukey–HSD test using SPSS Version 18.0 (IBM SPSS Statistics, Armonk, NY, USA). A probability value of $P < 0.05$ was considered significant.

Results

Hepatocyte morphology

Figure 2 shows the serial morphological changes in each group. On day 1 after dissemination, in the (-) group, hepatocytes had attached to the entire collagen-coated surface of the dish, while, in the 4+ group, the attachment area of the hepatocytes on the dish seemed smaller than that in the (-) group. On day 7, in the (-) group, hepatocytes became confluent on the dish, while, in the 4+ group, they formed a spheroid, resulting in a 3D configuration, which seemed to increase in size. On day 14, in the (-) group, hepatocytes tended to lose their specific configuration, showing a spindle shape, while, in the 4+ group, hepatocytes maintained their spheroid shape.

Hepatocyte-specific function

In order to measure the specific function of hepatocytes, albumin production was analyzed. Figure 4 showed the amount of albumin production measured by ELISA. There was no significant difference between the groups on day 3. On day 7, the albumin concentration in the culture medium of the 4+ group was significantly higher than in the other groups ($P<0.001$). On the other hand, in the (-) group, the albumin concentration was significantly lower than in the other groups excluding 1+ ($P<0.05$). On day 14, the albumin concentration of the 4+ group was significantly higher than
Figure 2. Phase-contrast micrographs of hepatocyte configurations on non-polymer (-) and PIPAAm (1+ - 4+) surfaces at 1, 7, and 14 days of culture.

Figure 3. The results of transmission electron microscopy (TEM) on day 7. Stress fibers (arrow) and oil drops(※) were seen in hepatocytes in the (-) group. In the 4+ group, there were intercellular bile canaliculi (BC), tight junctions(triangle), and peroxisomes(*).
in the other groups excluding 3+ (P<0.05). Alternatively, in the (-) group, the albumin concentration was significantly lower than in the other groups (P<0.05). Serially, in the (-) group, the albumin concentration decreased with time. Also, in the 1+ group, employing a regular, commercially available Upcell® dish, albumin synthesis reduced after peaking on day 3. However, its reducing tendency was slower than in the (-) group. In the 3+ 4+ group, albumin production remained constant from day 3 until day 14.

Discussion

Up until the present, marked efforts have been made to maintain the specific functions of primary cultured hepatocytes. Among them, the formation of a spheroid configuration of hepatocytes has been regarded as promising (13-17). Previously, hepatocyte spheroids were constructed using Nanopillar sheets, hollow fibers, Matrigel, TiO2 gel surfaces, oscillatory agitation, agarose-coated dishes, a spinner-mediated method, a galactosylated nanofiber scaffold, liver-derived proteoglycan-coated surfaces, and suspension cultures. In many of these previously reported methods for spheroid formation, there were problems such as the complexity of culture methods, coexistence of other elements when the spheroid was collected, and being time-consuming.

In this study, we explored the possibility of producing hepatocyte spheroids using a newly developed, temperature-dependent PIPAAm. When PIPAAm is 32°C, cells attach to the surface. On the other hand, when PIPAAm is increased in thickness, it is difficult for hepatocytes to attach to the surface (4). In addition, various thicknesses of PIPAAm were studied to maintain the specific function of hepatocytes for a longer time period.

Initially, a pilot study was performed to select various coating materials, including type I rat tail collagen, mouse collagen IV, and human fibronectin. Subsequently, three concentrations of PIAAm (300, 600, and 900 µg/ml) were studied. It was found that collagen I or fibronectin coating with 600 µg/ml of PIAAm for three hours was the optimal condition to induce excellent cell adherence and detachment.

There was no difference in the number of attaching cells, although the contact area of cells increased/decreased depending on the amount of polymer. The hepatocytes progressed horizontally if the amount of polymer was low. On the other hand, hepatocytes maintained their 3D spheroid structure with an increase in the amount of polymer.

It was also suggested that focal adhesion kinase (FAK) activation induces cells to form spheroids in the presence of abundant collagen, resulting in a higher hepatocyte-specific function (18).

Our method of spheroid formation has the following advantages: 1, spheroids can be formed in a static culture system; 2, spheroids can be harvested without any enzymatic reaction, which could damage cell integrity. It is necessary to evaluate the hepatocyte-specific function after trans-

Figure 4. The amount of albumin production measured by ELISA. Asterisk indicates p<0.05 vs (-). Pound sign indicates p<0.05 vs (-).
planting the spheroids. After having formed cell-to-cell contact, cell spheroids remained detached above the dish, and continued to show spheroid formation.

There are several limitations of this study, including the unavoidable flushing of cells on the temperature-responsive culture plate when media are exchanged. Moreover, it is necessary to develop a coating agent for the incubation system that does not use FBS for clinical application in the future.

In conclusion, hepatocyte-derived spheroids could be produced by long-term culture on the temperature-responsive culture plate. The spheroids maintained their 3D structure and the function of hepatocytes. The above-mentioned method can be effectively employed according to the use and site of the graft. In the future, transplants and metabolic examinations can be expected using the developed spheroids.

Acknowledgements

The authors thank Professor Teruo Okano, Kazuo Ohashi, Kohei Tatsumi, and Rie Utoh (Institute of Advanced Biomedical Engineering and Science Tokyo Women’s Medical University) for teaching many techniques to treat hepatocytes and use UpCell®.

References


