Formation of cylindrical multicellular aggregate (cylindroid) and expression of liver specific functions of primary rat hepatocytes

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Abstract

In our studies of the development of a hybrid artificial liver, we investigated the formation of cylindrical multicellular aggregate (cylindroid) of primary rat hepatocytes on a pressed sheet of polyurethane foam (pressed-PUF) as a culture substratum. Hepatocytes formed cylindroids by attaching to a pressed-PUF surface, peeling off from the surface and aggregating. The diameter and length of most cylindroids were approximately 200–500 μm and 500 μm –2 mm, respectively. The activities of liver specific functions (albumin secretion and ammonia metabolism) of hepatocyte cylindroids were equivalent to or higher than those of hepatocyte spheroids. These results suggest that hepatocyte cylindroids can maintain highly differentiated functions longer than hepatocyte spheroids, and that a PUF/cylindroid culture may be effective to develop of a hybrid artificial liver.

Abbreviations: PUF, polyurethane foam

Introduction

To save fulminant hepatic failure patients, a hybrid type artificial liver using primary hepatocytes culture technology has been developed by many researchers (Rozga et al., 1993; Gerlach et al., 1994). To develop a hybrid artificial liver as a bioreactor, maintaining differentiated functions of isolated hepatocytes and immobilizing the cells in an artificial matrix is very important. However, the full maintenance of the differentiated functions of hepatocytes *in vitro* is still difficult. For example, in most cases, albumin secretion activity gradually decreases when the cells are cultured in a monolayer (Crane et al., 1977).

Recently, it has become clear that hepatocytes, which has the form of a tissue-like structure (multicellular aggregates), express highly differentiated functions. A technology that makes individual cells reorganize *in vitro* has been tested. Koide et al. (1990) reported that primary rat hepatocytes form spherical multicellular organoids (spheroids) using proteoglycan substrates derived from liver reticulin fibers.

We reported that primary rat hepatocytes form multicellular spheroids in the pores of polyurethane foam (PUF) as a culture substratum, and that liver specific functions can be maintained for more than two weeks (Matsushita et al., 1991). The mechanism of spheroid formation in the pores of PUF is not clear. We conjectured that the spheroid forming factors were, (1) accumulation of autocrine substances from cells in the pores of PUF, and (2) the geometrical architecture of PUF.

In this study, we investigated the hepatocyte morphology and the expressions of liver specific functions by investigating the effect of the geometrical architecture of PUF (macroporous structure) using pressed-PUF (no pore structure) as a culture substratum.

Materials and methods

Materials

Unless otherwise noted, all chemical and cell culture

reagents were purchased from Sigma Chemical Co. (U.S.A.). Culture dishes and type I collagen – coated dishes were purchased from IWAKI GLASS (Japan).

PUF and pressed-PUF as a culture substratum

PUF and its raw materials were kindly donated by INOAC Co. Ltd. (Nagoya, Japan). It has a sponge-like macroporous structure with each pore made up of smooth thin films and thick skeletons (Matsushita et al., 1990). To prepare a stationary culture, a block of PUF was cut into flat plates ($1 \times 25 \times 25$ mm). Before use, the cut PUF plates were submerged and washed in distilled water to remove chemical contaminants and were autoclaved to sterilize them. Then, the PUF substrates were soaked in a culture medium prior to cell inoculation.

The pressed-PUF was a film-like PUF with a smooth surface. The raw materials of PUF were mixed, and the PUF was pressed in the process of foaming (Figure 1). To prepare the stationary culture, the pressed-PUF was cut into discs of 35 mm diameter, followed by the same procedure as for the PUF.

Hepatocyte culture

Hepatocytes were isolated from the liver of an adult male Wistar rat (7–8 weeks, 200–215 g) by the liver perfusion method using 0.05% collagenase (WAKO CHEMICALS, Japan). The isolated hepatocytes were dispersed in a hormone-defined medium (HDM) [Williams' medium E supplemented with $10 \mu\text{g ml}^{-1}$ insulin, $0.1 \mu\text{M CuSO}_4 \cdot 5\text{H}_2\text{O}$, $3 \mu\text{g ml}^{-1} \text{H}_2\text{SeO}_3$, $50 \text{ pM ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 50 ng ml^{-1} EGF (FUNAKOSHI, Japan), $50 \mu\text{g ml}^{-1}$ linoleic acid, $58.8 \mu\text{g ml}^{-1}$ penicillin, $100 \mu\text{g ml}^{-1}$ streptomycin]. Cells were inoculated on to each pressed-PUF plate that was put into a culture dish (diameter: 35 mm) at a density of $1.04 \times 10^5 \text{ cells cm}^{-2}$ ($5.0 \times 10^5 \text{ cells ml}^{-1}$ -medium), and were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C . The medium was exchanged 4 and 24 h after cell inoculation, and then every 48 h.

To compare the activities of liver specific functions of cultured hepatocytes on pressed-PUF, a monolayer culture and PUF/spheroid culture were performed in parallel studies. For the monolayer culture, hepatocytes were inoculated at a density of $5.2 \times 10^4 \text{ cells cm}^{-2}$ ($2.5 \times 10^5 \text{ cells ml}^{-1}$ -medium) on the bottom of a type I collagen – coated dish (diameter: 35 mm). The medium was exchanged as described above. For

the PUF/spheroid culture, hepatocytes were inoculated at a density of $3.0 \times 10^6 \text{ cells per PUF plate}$, which was put into a culture dish. After 4 h of cell inoculation, the PUF plates were transferred to another culture dish containing fresh medium. Then, the medium was exchanged after 24 h of cell inoculation and then every 48 h.

Morphological analysis

The morphology of hepatocytes inoculated on to pressed-PUF was observed using a phase contrast microscope with a video camera and a time lapse video. The culture dish was put on the microscope stage and was maintained in 5% CO_2 , 95% air, at 37°C .

Liver specific functions (albumin secretion and ammonia metabolism)

The albumin concentration in the culture medium was measured using an enzyme-linked immunosorbent assay. When we evaluated the ammonia metabolic activity, the culture medium was changed to a fresh medium containing $1 \text{ mM-NH}_4\text{Cl}$, and the ammonia concentration was measured at 6 h after incubation using a commercial kit (Ammonia test Wako: WAKO CHEMICALS, Japan). For each normalized albumin secretion and ammonia metabolic activity, the rates were divided by the nuclei number obtained by the nucleus counting method of van Wezel (Wezel, 1967).

Statistical analysis

Data represented the mean $\pm$ standard derivation of six experiments from two independent cell preparations. Only the data at 15 days of culture were shown from three experiments. Statistical significances were determined by the Student's *t*-test. A *p* value less than 0.05 was considered statistically significant.

Results

Cylindroid formation of adult rat hepatocytes

Hepatocytes inoculated on to pressed-PUF formed cylindrical multicellular aggregates (cylindroids) at random on the pressed-PUF by day 7 of the culture (Figure 2d). The size of the cylindroids varied and their diameter and length were mostly approximately $200\text{--}500 \mu\text{m}$ and $500 \mu\text{m--}2 \text{ mm}$, respectively.

A

Isocyanate : Crude MDI
(Diphenylmethane diisocyanate)

Polyol : Glycerin + Ethylene oxide + Propylene oxide

B

Figure 1. Preparation of pressed-PUF. (A) Main raw materials of PUF; (B) Schema of preparation of pressed-PUF.

The cylindroids formed by the following steps. When freshly isolated hepatocytes were inoculated on to pressed-PUF, they initially attached to the pressed-PUF surface, and spread out to form a monolayer (Figure 2a). Subsequently, the hepatocytes spontaneously peeled off from the pressed-PUF surface, and

formed small cell aggregates or large strand-like aggregates (Figure 2b, c). The small aggregates grew to form spheroids after 1–3 days of culture. On the other hand, the large aggregates grew, sometimes incorporating previously formed spheroids, to form into cylindroids at 5–7 days of culture (Figure 2d).

Figure 2. Photographs of primary rat hepatocytes in a PUF/cylindroid culture. Several specific patterns inside the pressed-PUF are shown (arrows). Hepatocytes inoculated on to pressed-PUF attach to the surface and form a monolayer in 1 day after inoculation (A). Hepatocytes spontaneously peel off from the surface and form many irregular cell aggregates in 3 days (B), and the cell aggregation makes progress with time (C). The cell aggregation grows to form multicellular cylindroids in about 7 days (D). H : hepatocytes. Bar: 200 μm (A) and 500 μm (B), (C) and (D).

Liver specific functions of hepatocyte cylindroids

To evaluate the activities of liver specific functions of hepatocytes inoculated on to pressed-PUF (hepatocytes in a PUF/cylindroid culture), the activities of albumin secretion and ammonia metabolism were compared with those of PUF/spheroid and monolayer cultures (Figures 3 and 4).

Figure 3 shows the activity of albumin secretion. At day 1 of the culture, the albumin secretion rate in the monolayer culture was significantly higher than that of the two other cultures (PUF/cylindroid and PUF/spheroid cultures) ($65.3 \pm 25.2 \mu\text{g } 10^{-6} \text{ nuclei day}^{-1}$). Following this, the albumin secretion activity in the monolayer culture rapidly decreased and disappeared by 15 days of culture. On the other hand, the activities in the PUF/cylindroid and PUF/spheroid cultures were maintained for at least 15 days of culture. After 7 days of culture, these activities were

significantly higher than that of the monolayer culture, and at 15 days of culture the activity in the PUF/cylindroid culture tended to be higher than that of the PUF/spheroid culture ($27.6 \pm 10.1 \mu\text{g } 10^{-6} \text{ nuclei day}^{-1}$).

Figure 4 shows the activity of ammonia metabolism. The metabolic rate of all three cultures continuously decreased for 15 days, and the activities in the monolayer and PUF/spheroid cultures disappeared by 15 days of culture. However, the activity in the PUF/cylindroid culture was maintained for at least 15 days of culture. At 15 days of culture, the activity in the PUF/cylindroid culture was significantly higher than that of the monolayer culture ($0.043 \pm 0.00466 \mu\text{mol } 10^{-6} \text{ nuclei h}^{-1}$).

Figure 3. Comparison of albumin secretion rates among PUF/cylindroid, PUF/spheroid and monolayer cultures. The data represent the mean $\pm$ standard deviation of six experiments from two independent cell preparations (only the data at 15 days of culture are shown from three experiments). At day 1 of culture, the activity in the monolayer culture is significantly higher than that of the two other cultures. After 7 days of culture, the activities in the PUF/cylindroid and spheroid cultures are significantly higher than that of the monolayer culture, and at 15 days of culture, the activity in the PUF/cylindroid culture tends to be higher than that of the PUF/spheroid culture (no significant difference). —○—: PUF/cylindroid culture; —△—: PUF/spheroid culture; —●—: monolayer culture.

Discussion

When the immobilized cell number in the PUF/spheroid stationary culture was more than 2×10^6 cells cm^{-3} —PUF, spheroid formation in the pores of the PUF was not observed. In this case, because the specific surface area ($\text{cm}^2 \text{ cm}^{-3}$) of PUF was $109 \text{ cm}^2 \text{ cm}^{-3}$, the maximal cell density of the surface that can form spheroids was 1.83×10^4 cells cm^{-2} . This cell density was one third the density at which hepatocytes cover the surface confluent. In this study, the inoculum cell density of pressed-PUF was 1.04×10^5 cells cm^{-2} , which was six times as much as the maximal cell density in the PUF/spheroid culture. However under such a highly dense cell condition, hepatocytes formed a tissue-like structure, i.e. cylindroid. This result shows that multicellular aggregates can be formed under highly dense conditions if oxygen and nutrient supplies are sufficient for the hepatocytes to survive. In a preliminary trial, we found that hepatocytes formed

only spheroids when the inoculum cell density on pressed-PUF was 2.6×10^4 cells cm^{-2} , and the hepatocytes did not form a tissue-like structure when the inoculum cell density was 2.08×10^5 cells cm^{-2} . This suggests that the shape of the multicellular aggregates (e.g. spheroid or cylindroid) depends on the cell density of the surface, and that the culture system should be improved under a high cell density condition with an inoculum cell density of more than 2.08×10^5 cells cm^{-2} . Further studies are needed to clarify the relationship of the shape of the aggregates and the inoculum cell density.

Hepatocyte reorganization and spheroid formation on various substrata have been reported (Roberts et al., 1993; Peshwa et al., 1996). In many cases, hepatocyte spheroids are formed by cell attachment, peeling and aggregation. In this study, we demonstrated that hepatocyte cylindroids form by the same steps as for spheroid formation. The mechanism of multicellular aggregate formation is not clear. We found in the pores

Figure 4. Comparison of ammonia metabolic rates among PUF/cylindroid, PUF/spheroid and monolayer cultures. The data represent the mean $\pm$ standard deviation of six experiments from two independent cell preparations (only the data at 15 days of culture are shown from three experiments). The activities in the monolayer and PUF/spheroid cultures disappear by 15 days of culture. However, the activity in the PUF/cylindroid culture is maintained for at least 15 days of culture and at 15 days of culture, the activity in the PUF/cylindroid culture is significantly higher than that of the monolayer culture. —○—: PUF/cylindroid culture; —△—: PUF/spheroid culture; —●—: monolayer culture.

of PUF that proteoglycan secreted by cultured hepatocytes accumulates and adsorbs to the PUF surface (data not shown), and we conjecture that the geometrical architecture (macroporous structure) of PUF is important for spheroid formation. But hepatocytes formed spheroids or cylindroids on pressed-PUF (no pore structure). We suggest that the surface properties of PUF (ex. hydrophobicity of surface) are important for multicellular aggregate formation. Previously, we reported that spheroids of primary rat, dog and porcine hepatocytes form rapidly by the different hydrophobicity of the PUF surface (Ijima et al., 1998).

With the expression of liver specific functions of cultured hepatocytes, the activities of albumin secretion and ammonia metabolism in the PUF/cylindroid culture were equivalent to those of the PUF/spheroid culture. Moreover, at 15 days of culture, although no significant differences were between two cultures, these activities in the PUF/cylindroid culture tended to be higher than those of the PUF/spheroid culture. These results suggest that hepatocyte cylindroids

maintain highly differentiated functions longer than hepatocyte spheroids, and a PUF/cylindroid culture may be effective to develop a hybrid artificial liver.

Conclusions

Hepatocytes inoculated on to pressed-PUF formed cylindroids, and the hepatocyte cylindroids showed highly differentiated functions and formed a high cell density culture. Hepatocyte cylindroids may be useful to develop a hybrid artificial liver in the future.

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