Desalination and Water Treatment
www.deswater.com

17 (2010) 227–232
May

1944-3994/1944-3986
doi: 10.5004/dwt.2010.1722

Hepatocyte spheroids formed on rubbed polyimide membrane for cell transplantation

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Received 23 July 2009; accepted 22 November 2009

ABSTRACT

In this study, we have prepared a rubbed fluorinated polyimide membrane using a rubbing machine with a rubbing cloth and formed hepatocyte spheroids on the rubbed membrane. The re-seeding of the spheroids, that is, spheroid building, enhanced the biochemical functions of the spheroids by optimization of the culture conditions. Furthermore, the co-culture of the spheroids with endothelial cells enhanced the spheroid function. To increase the specific function without co-culture, we attempted to transplant the hepatocyte spheroid into rat spleen. The transplanted spheroid without dispersion was observed by hematoxylin-eosin staining. Moreover, immunostaining proved that the resulting spheroids in the spleen expressed liver-specific antigen on cell surface. These results suggest that the hepatic spheroid formed on the rubbed polyimide membrane succeeded to be transplanted.

Keywords: Polyimide membrane; Rubbing; Hepatocyte spheroid; Cell transplantation; Endothelial cell

1. Introduction

Controlling the cell morphologies of a material is of interest with regard to tissue cultures, because the morphologies are closely related to cell functions. The cell spheroids, which are a spherical mass composed of many cells and extracellular matrices, have been used in the research areas of tissue engineering or cell chips, because they appear to mimic not only the morphology but also the physiological functions of cells in living tissue and organs, unlike the conventional two-dimensional monolayer culture of cells [1–5]. The spheroid is well-known to sustain viability for extended culture periods and maintain high levels of cell functions when compared with those of cells as monolayers. However, it has been difficult to prepare multicellular spheroids from cells, which do not easily aggregate, and, in addition, to produce many spheroids from the cell culture.

On the other hand, micropatterning is also becoming increasingly popular for the development of improved biomaterials and medical devices. In particular, surface microfabrication techniques have been discovered and developed to create materials for regulating cell functions [6–8]. Several techniques have been investigated for fabricating micropatterns on surfaces, including the use of conventional photoresist lithography, photochemistry, and self-assembled monolayers. However, the processes to fabricate the

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Presented at the Fifth Conference of the Aseanian Membrane Society Aseania 2009 "Recent Progress in Membrane Science and Technology", 12–14 July 2009, Kobe, Japan.

patterning for biomaterials and medical devices are very complex.

We have clarified that polyimides containing a fluorinated group are promising materials for medical devices [9,10]. Recently, we succeeded in fabricating a micropattern on a fluorinated polyimide surface using a rubbing method by high pressure and reported a specific interaction between the cells and the fluorinated polyimide surface modified by the rubbing [11,12]. We demonstrated that the morphologies of rat skin fibroblast cells attached to a rubbed fluorinated polyimide film were three-dimensional multicellular spheroids, while the cells on the unrubbed film were of the two-dimensional monolayer type. The rubbing method is a simple process for the formation of multicellular spheroids when compared with conventional methods, which are the liquid-overlay technique with a non-adhesive substrate and the semi-adhesive substrate method, and, in addition, for the formation of a patterned surface when compared with conventional techniques, which are photoresist lithography, photochemistry, and self-assembled monolayers.

We have already reported that the cell adhesion on a rubbed fluorinated polyimide membrane prepared by a rubbing machine made in our laboratory. We easily fabricated an ordered pattern surface using the machine, because the machine can exactly control the experimental parameters, such as rubbing velocity and pressure, in order to prepare the rubbed surface, when compared with the manual rubbing method. We selected the fluorinated polyimide as the cell culture material, and rat primary hepatocytes were used for the *in vitro* studies [13].

In this study, we investigate the enhancement of the biochemical function of the resulting spheroids formed on rubbed polyimide membrane under various culture conditions including co-culture with endothelial cells. Subsequently, we carried out the engineered implant of the resulting spheroid to spleen *in vivo*.

2. Materials and methods

2.1. Materials

2,2′-Bis(3,4-dicarboxyphenyl)hexafluoropropane dianhydride (6FDA) was purchased from the Central Glass Co., (Saitama, Japan) and purified by sublimation prior to use. 2,2′-Bis(4-aminophenyl) hexafluoropropane (6FAP) was purchased from the Central Glass Co., (Saitama, Japan) and recrystallized twice from an ethanol solution prior to use.

Fluorinated polyimide, 6FDA-6FAP, was synthesized by chemical imidization of the poly(amic acid) precursors as reported in a previous paper [14]. The molecular weight of the polyimide was 4.2×10^5. The polyimide films were carefully prepared by a solvent-casting method from a tetrahydrofuran solution on a glass plate to obtain a uniform surface and were cured at 150°C for 15 h. The obtained films were optically clear.

2.2. Rubbed polyimide film

The rubbing was carried out by a rotating cylinder covered with a rubbing cloth at a constant velocity. The cloth was Bemcot. The rubbing velocities of the cylinder were 20 mm/s. The rubbing pressures added to the cylinder were 2.5, 5.0, and 7.5 MPa. The substrate temperature was 20°C.

2.3. Cell culture

Primary hepatocytes were isolated from Sprague Dawly rats (240-–250 g) using the collagenase perfusion method [15]. The isolated cells were filtered four times through a 100 μm cell strainer and centrifuged at 50G for 3 min. The viability of the isolated hepatocytes determined using Trypan Blue was more than 70%. The primary hepatocytes were cultured in Williams' E medium containing 1% antibiotics, 10% fetal bovine serum, and 2.2 g/L $NaHCO_3$. The cells were maintained in a humid, 5% CO_2 incubator at 37°C. Bovine aorta endothelial cells (BAECs) were used below 10th passage.

For morphological observation of the cells, the cells adhering to the membrane were photographed by a digital camera coupled to an inverse phase contrast microscope (Nikon, ECLIPSE TE300, Tokyo, Japan).

2.4. Measurement of albumin concentration

The amounts of rat albumin in the medium were determined by enzyme-linked immunosorbent assay (ELISA) with purified albumin, antibody to rat albumin and peroxidase-conjugated antibodies (Rat albumin ELISA kit AKRAL-120, Shibayagi, Gunma, Japan). The albumin concentration from the hepatocytes was quantified at 490 nm with an ELISA reader (BIO-RAD, Model 550, CA, U.S.A.) and was determined from a standard curve. Data are given as mean $\pm$ S.D. Duplicate wells were averaged for each sample.

2.5. Spheroid transplantation

Primary rat hepatocytes were cultured on the rubbed fluorinated polyimide membrane prepared by

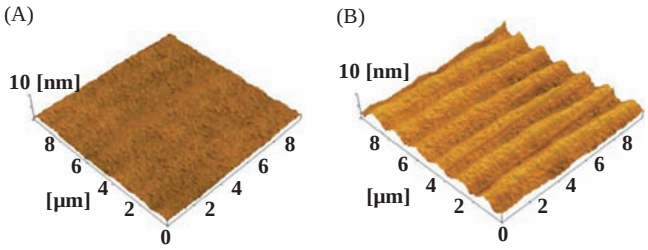

Fig. 1. AFM images of polyimide surfaces: (A) Unrubbed surface and (B) rubbed surface.

a rubbing machine. Cell morphology was observed by phase-contrast microscope and albumin secretion (liver specific function) was determined by enzyme-linked immunosorbent assay. The spheroids which were cultured for 4 d were harvested and transplanted into rat spleen *in vivo*. After 2 W, the resulting spleen was observed by histological and immunohistochemical analyses.

3. Results and discussion

3.1. Spheroid culture under various conditions

As shown in Fig. 1, as described in our previous paper [13], the morphology formed on the rubbed polyimide surface indicated a relief pattern such as grooves, which was confirmed by AFM. The spheroid formation was carried out on two sheets of rubbed polyimide membrane for 3 d (4.0×10^5 cells/mL). Subsequently, we have detached the resulting spheroids and chosen three kinds of spheroid culture methods

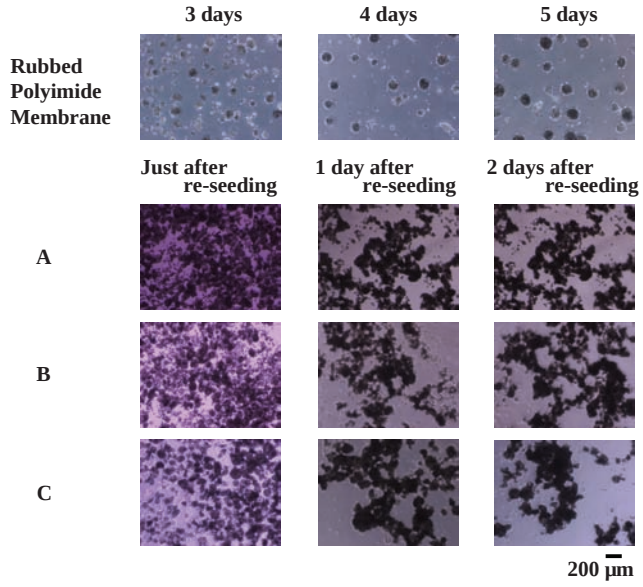

Fig. 2. Phase-contrast micrographs of rat hepatocyte spheroids under various conditions.

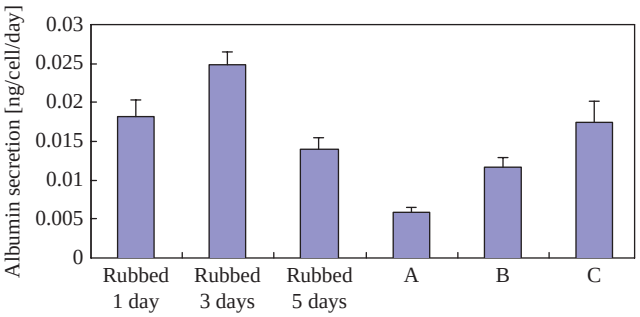

Fig. 3. Albumin secretion of rat hepatocyte spheroids under various conditions after re-seeding for 2 d.

as follows: (A) The rubbed polyimide membrane with 9 mm diameter was fixed on the bottom of the culture dish with 10 mm diameter. The spheroids were incubated in 1 mL of medium. (B) The rubbed polyimide membrane with 9 mm diameter was fixed on the bottom of the culture dish, whose height was half of method A, with 10 mm diameter. The spheroids were incubated in 0.5 mL of medium. Then, 3 mL of medium covered the top of the culture dish. (C) The rubbed polyimide membrane with 1.5 cm × 1.5 cm square was fixed on the bottom of the culture dish. Then, the cylinder with 10 mm diameter was fixed on the bottom of the culture dish, followed by the incubation of the spheroids in 1 mL of medium. After 1–2 h incubation with 1 mL of additional medium, the cylinder was removed.

The morphology of the resulting spheroids is shown in Fig. 2. Under any method, spheroid formation was observed after re-seeding for 2 d. As compared with method A, however, larger spheroids were observed under methods B and C. The surface of the larger spheroids was smooth. To determine the function of the spheroids, we examined the albumin secretion of the resulting spheroids after re-seeding 2 d, as shown in Fig. 3. The highest secretion of albumin was observed under method C, which was higher secretion than spheroid culture without re-seeding. These results suggest that oxygen consumption is important factor because of the oxygen supply from all direction except the polyimide membrane surface under method C. Under methods A and B, there is no oxygen supply from the side direction because of the obstacle by the wall of the culture dish. The removal of the cylinder under method C is considered to overcome the obstacle by the wall.

3.2. Spheroid culture under various amounts of medium

To increase the oxygen supply, we increased the amount of the medium under method C. Namely, the

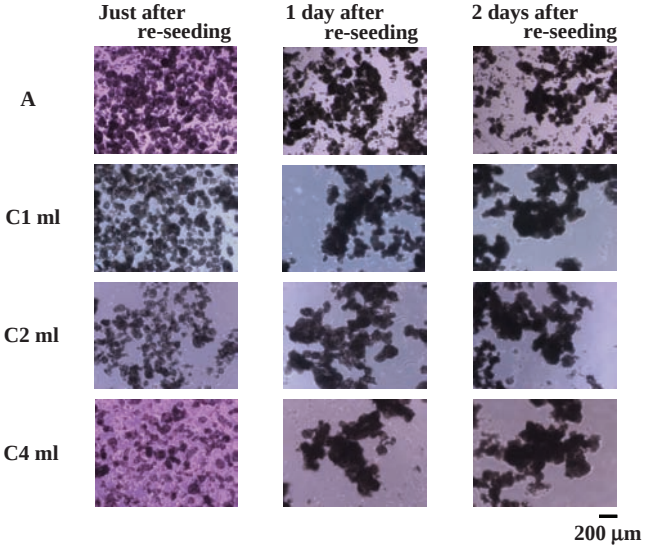

Fig. 4. Phase-contrast micrographs of rat hepatocyte spheroids under various amounts of medium.

rubbed polyimide membrane with 1.5 cm × 1.5 cm square was fixed on the bottom of the culture dish. Then, the cylinder with 10 mm diameter was fixed on the bottom of the culture dish, followed by the incubation of the spheroids in 1 mL of medium. After 1–2 h incubation with 0, 1 or 3 mL of additional medium, the cylinder was removed. As above, the amount of the total medium was 1, 2 or 4 mL.

Fig. 4 shows the morphology of the resulting spheroids. Large spheroid formation was observed in any amount of medium after re-seeding for 2 d. However, there is no significant difference of the morphology. To determine the function of the spheroids, therefore, we examined the albumin secretion of the resulting spheroids after re-seeding 2 d, as shown in Fig. 5. The highest secretion of albumin was observed in 4 mL of medium. These results suggest that the oxygen supply as well as essential element of the medium increased to enhance the spheroid function.

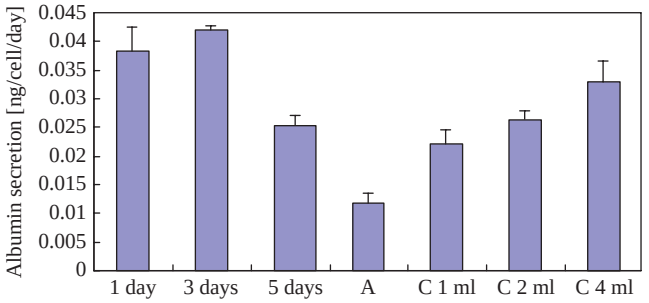

Fig. 5. Albumin secretion of rat hepatocyte spheroids under various amounts of medium after re-seeding for 2 d.

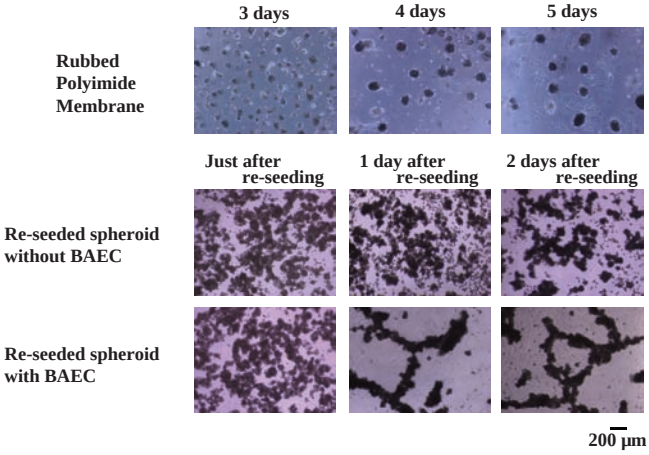

Fig. 6. Phase-contrast micrographs of rat hepatocyte spheroids co-cultured with endothelial cells under method A conditions.

3.3. Co-culture of the spheroids with endothelial cells

To increase the spheroid function more and to imitate the native structure of liver, we co-cultured the spheroids with endothelial cells (1.5×10^5 cells/mL). The morphology of the resulting spheroids after the co-culture under method A is shown in Fig. 6. The co-cultured spheroids were rapidly formed after re-seeding for 1 day. Furthermore, the network-like structure of the co-cultured spheroids was observed after re-seeding for 2 day. These results suggest that the endothelial cells mediated and enhanced the spheroid–-spheroid interaction.

Fig. 7 shows the albumin secretion of the co-cultured spheroids after re-seeding for 2 d. The secretion of the co-cultured spheroid was lower than that without re-seeding. However, it should be noted that the co-cultured spheroids exhibited the higher secretion, as compared with the spheroids consisting of hepatocyte alone. These results suggest that the

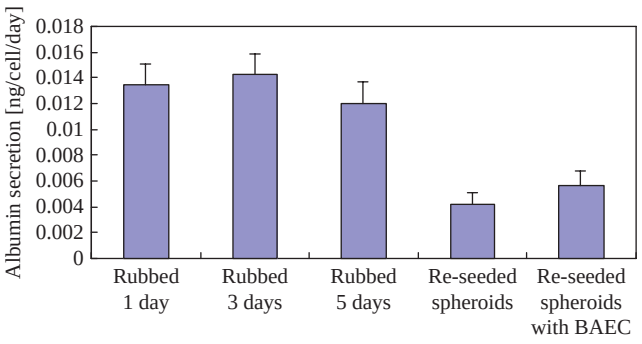

Fig. 7. Albumin secretion of rat hepatocyte spheroids co-cultured with endothelial cells under method A conditions after re-seeding for 2 d.

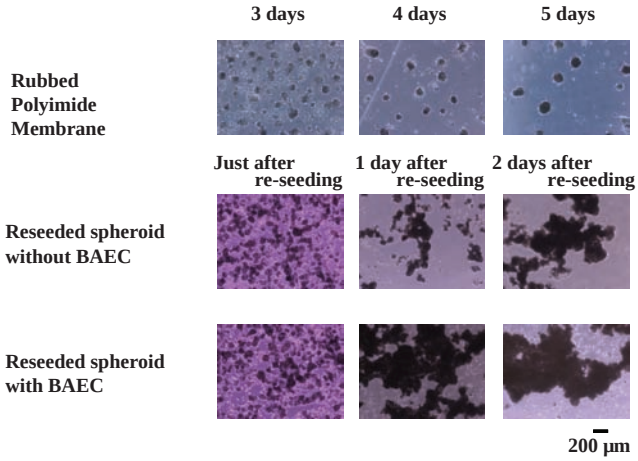

Fig. 8. Phase-contrast micrographs of rat hepatocyte spheroids co-cultured with endothelial cells under method C conditions.

effect of the co-culture diminished because of severe culture conditions (method A).

To improve the severe culture conditions, we co-cultured the spheroids under method C conditions. Fig. 8 shows the morphology of the resulting spheroids after the co-culture under method C. The rapid formation of spheroids was also observed after re-seeding for 1 d. Twice size of the spheroids was formed, as compared with that without endothelial cells, after re-seeding for 2 d. However, no network-like structure was observed. These results suggest that the increased function of the spheroids under method C enhanced the motility of resulting spheroids.

The albumin secretion of the co-cultured spheroids after re-seeding for 2 d is shown in Fig. 9. The albumin secretion of the spheroids increased by the co-culture with endothelial cells. Furthermore, the resulting secretion was higher than that of the spheroids without re-seeding. These results suggest that the endothelial cells enhanced the function of the spheroid under adequate culture conditions in view of the oxygen supply

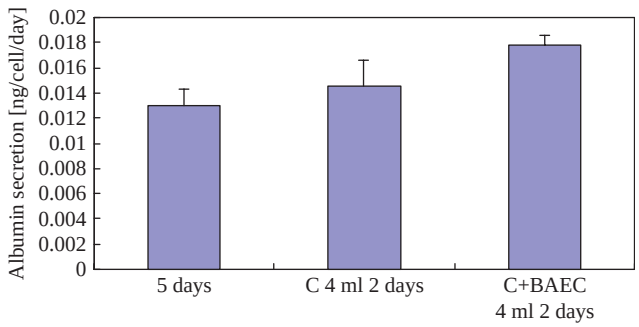

Fig. 9. Albumin secretion of rat hepatocyte spheroids co-cultured with endothelial cells under method C conditions after re-seeding for 2 d.

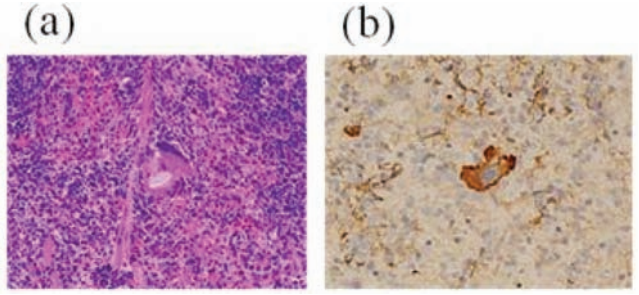

Fig. 10. Histological section of the rat spleen where a spheroid was transplanted. (a) Hematoxylin-eosin staining and (b) immunostaining with antibody for keration after re-seeding for 2 d.

as well as essential element of the medium. The enhancement may be attributed to the formation of the blood vessel structure to supply oxygen as well as nutrient. As another effect, laminin, one of the basement membrane components, which exists between hepatocytes and sinusoidal endothelial cells in the liver, is considered. The extracellular matrix is believed to play essential roles during *in vitro* culturing of primary hepatocytes in the control of differentiation and in the maintenance of tissue-specific functions [16]. Furthermore, enhancement of hepatic functions of random co-cultured hepatocytes were explained with deposition of extracellular matrix proteins between hepatocytes and non-parenchymal cells [17]. Therefore, it was supposed that hepatocytes with endothelial cells in the spheroids were placed in the structurally and microenvironmentally favorable conditions for hepatic function maintenance.

3.4. Transplantation of the spheroids into rat spleen

To increase the specific function by the induction of native endothelial cells, we attempted to transplant the spheroids of hepatocyte alone into rat spleen. As shown in Fig. 10a, the transplanted spheroids without dispersion was observed by hematoxylin-eosin staining. Furthermore, immunostainig proved that the resulting spheroids in the spleen expressed liver-specific antigen on the cell surface (Fig. 10b). These results suggest that the hepatic spheroids formed on the rubbed polyimide membrane succeeded to be transplanted.

4. Conclusions

In this study, we have prepared a rubbed fluorinated polyimide membrane using a rubbing machine with a rubbing cloth and formed hepatocyte spheroids on the rubbed membrane. The re-seeding of the

spheroids, that is, spheroid building, enhanced the biochemical functions of the spheroids by optimization of the culture conditions in view of the supply of oxygen as well as nutrient. Furthermore, the co-culture of the spheroids with endothelial cells enhanced the spheroid function by vascular-like structure or produced extracellular matrices. To increase the specific function without co-culture to induce native endothelial cells, we attempted to transplant the hepatocyte spheroid into rat spleen. The transplanted spheroid without dispersion was observed by hematoxylin-eosin staining. Moreover, immunostaining proved that the resulting spheroids in the spleen expressed liver-specific antigen on cell surface. The transplantation technique in this study is expected to be promising therapy for liver disease.

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