Special Issue

Multilayer rat hepatocyte aggregates formed on expanded polytetrafluoroethylene surface

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Received 9 January 1997; accepted 10 July 1997

Key words: ePTFE, hepatocytes, multilayer hepatocyte aggregates, porous membrane, primary culture

Abstract

Feasibility of using a macroporous membrane material, expanded polytetrafluoroethylene (ePTFE), for culturing hepatocytes on its surface was examined. Adult rat hepatocytes were attached to an ePTFE surface and cultured in a hormonally defined medium supplemented with or without fetal calf serum (FCS, 10%) or bovine serum albumin (BSA, 0.03–3%). When cultured in a FCS-supplemented medium, hepatocytes reorganized themselves into multilayer cell aggregates on an ePTFE surface. The morphological characteristics of hepatocytes were influenced by the modification of the ePTFE surface as well as the culture medium. Hepatocytes cultured on a polyvinylalcohol (PVA)-coated ePTFE surface formed many more multilayer cell aggregates than those cultured on an uncoated ePTFE surface. Such highly multilayered hepatocyte aggregates were also noted when the cells were cultivated in a BSA-supplemented medium. On the other hand, when cultured in a FCS- or BSA-free medium, hepatocytes formed cell monolayers on both PVA-coated and uncoated ePTFE surfaces as did the cells on a collagen-coated polystyrene surface. The hepatocytes in the aggregates exhibited high albumin expression capability and low DNA synthesis rate as compared with those in monolayer cultures. The multilayer hepatocyte aggregates, as immobilized on a PVA-coated ePTFE surface in a serum-supplemented medium, are shown to be not only morphologically, but functionally differentiated, and will provide us a model system for the development of a bioreactor using hepatocytes, particularly for a hybrid-type artificial liver.

Abbreviations: ePTFE, expanded polytetrafluoroethylene; FCS, fetal calf serum; BSA, bovine serum albumin; PVA, polyvinylalcohol

Introduction

To develop liver substitutes that restore, maintain or improve hepatic functions, a variety of hepatic tissue engineering systems have been studied using isolated hepatocytes. The growth and function of cultured hepatocytes are known to depend on the mode of cell to cell interaction and cell to substrate interaction.

Cultures on synthetic polymers in contrast to culture on a plastic surface have called increasing attention since the use of the former material has been shown to cause functional differentiation of epithelial cells

mimicking an *in vivo* condition (Baldwin and Saltzman, 1996).

An expanded polytetrafluoroethylene (ePTFE) membrane has a macroporous structure providing a high surface area to volume ratio and surface complexity, while it is chemically inert (Hirabayashi *et al.*, 1992). It has been studied as a candidate material for vascular substitute (Hirabayashi *et al.*, 1992). However, few studies on the use of ePTFE for culturing isolated hepatocytes on its surface have been done so far. In the present work, we examined ePTFE as a culture substrate in terms of the morphology and albumin

Figure 1. Phase contrast micrographs of hepatocytes cultured on various substrates. Hepatocytes were cultured for 1 day in a FCS-supplemented (a, c and e) or -free medium (b, d and f). (a,b) N-ePTFE, (c,d) P-ePTFE, (e,f) Ps.

expression of rat hepatocytes cultures on its surface *in vitro*. The results will provide information on its applicability in tissue engineering such as for use in a hybrid-type artificial liver device.

Materials and methods

Primary culture of hepatocytes

Parenchymal hepatocytes were isolated from 150–250 g male Wistar rats by perfusion of the liver *in situ*

Figure 2. Scanning electron microscopy of hepatocytes cultured on PVA-coated ePTFE surfaces. Hepatocytes were cultured for 4 days in a FCS-supplemented (a, c) or -free medium (b).

with collagenase (Seglen, 1976). Dead cells and debris were removed by centrifugation in an isodensity Percoll (Pharmacia LKB Biotechnology, Uppsala, Sweden) solution. Viability at plating ranged from 88 to 95%. Hepatocytes were dispersed into a hormonally defined medium (HDM)(Enat *et al.*, 1984) which was composed of William's E medium supplemented with dexamethasone (10^{-6} M)(Sigma), insulin (10^{-7} M)(Wako Chemical, Osaka, Japan), epidermal growth factor (25 ng/ml)(Austral Biologicals, Lake Placid, NY) and 100 U/ml penicillin/streptomycin (Wako) and were plated onto 30-mm Millicell culture plate inserts (Millipore, Bedford, MA) attached with ePTFE membranes as mentioned below. The cells were plated at a density of 1×10^5 cells/cm² unless stated otherwise. The inserts with these membranes were placed in 6 well polystyrene plates (Iwaki, Tokyo, Japan). One and 1.5 ml of culture medium were added to the

upper and the lower chamber of the plates, respectively. Type I collagen-coated plastic plates (Ps; Iwaki, Tokyo, Japan) were used as control. The cells were cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. The culture medium was changed routinely every 2 days. To test the effect of fetal calf serum (FCS; Gibco BRL, Gaithersburg, MD) and bovine serum albumin (BSA; Wako) on the formation of multilayer cell aggregates, hepatocytes were dispersed into HDM with or without 10% FCS or 0.03–3% of BSA and cultured as mentioned above.

Membranes

The polyvinylalcohol (PVA)-coated (hydrophilic) (P-ePTFE) and uncoated (hydrophobic) ePTFE membranes (N-ePTFE) were provided by Sumitomo Denko, Co., Osaka, Japan. The fibril length and thickness of

Figure 3. Phase contrast micrographs of hepatocytes cultured on PVA-coated ePTFE surfaces. Hepatocytes were cultured for 4 days in BSA-containing media. (a) 3% BSA, (b) 0.3% BSA, (c) 0.03% BSA.

ePTFE membranes used were approximately 30 μm and 100 μm , respectively. PVA in the membranes was cross-linked by electronic beam irradiation by the manufacturer. The presence of PVA in PVA-coated ePTFE surfaces was confirmed by FTIR-ATR.

Scanning electron microscopic observation

The culture samples were fixed with 2% glutaraldehyde, dehydrated through a graded series of ethanol,

Figure 4. Sections of multilayer hepatocyte aggregates formed on PVA-coated ePTFE surfaces and stained with hematoxylin-eosin. Hepatocytes were cultured for 3(a), 4(b) and 8(c) days in a FCS-supplemented medium. Hematoxylin-eosin.

and finally soaked in isoamyl acetate. The samples were dried with a critical dryer (HITACHI HCP-1), coated with Au using an Eiko Ion coater (Model IB-5), and then observed with a HITACHI S-4500 scanning electron microscope.

DNA synthesis

DNA synthesis was measured by the incorporation of [^3H]-thymidine. hepatocytes were plated at a low cell density (2×10^4 cells/ cm^2) and cultured for 48 h. Eight

Figure 5. LDH leakage from hepatocytes cultured on various substrates. Hepatocytes were cultured for 8 days in a FCS-supplemented medium. Each value is the mean of two experiments.

h before the end of the incubation period, the cells were incubated with [3 H]-thymidine ($1.25 \mu\text{Ci ml}^{-1}$). The cell layer was washed twice in cold PBS and proteins precipitated using 10% ice cold trichloroacetic acid. The culture dishes were then washed twice with 70% and 100% ethanol and DNA was dissolved in 0.5N sodium hydroxide solution at room temperature for 30 min. After neutralization in 0.5N hydrochloric acid solution, the radioactivity of samples was counted by scintillation counter.

The level of lactate dehydrogenase

The level of lactate dehydrogenase (LDH) in culture medium was determined by the LDH CII test kit (Wako).

Enzyme-linked immunosorbent assay

Albumin secreted into the culture medium was assessed by an enzyme-linked immunosorbent assay (ELISA) using anti-rat albumin rabbit antibody and horseradish peroxidase-conjugated anti-rat albumin rabbit antibody (Cappel, Cochranville, PA) as described before (Tokiwa *et al.*, 1996). The limits of detection in ELISA were 1.0 ng/tube and the medium including 10% FCS had no detectable albumin.

Figure 6. DNA synthesis by hepatocytes cultured on various substrates. Hepatocytes were plated at 2×10^4 cells/cm 2 and cultured for 2 days in a FCS-supplemented (FCS+) or -free (FCS-) medium. DNA synthesis was measured as described in Materials and Methods. Each value is the mean $\pm$ SD of three experiments.

Immunocytochemical staining

The culture samples were fixed with neutral buffered 10% formalin and then processed to paraffin wax for sectioning. Immunocytochemistry was performed by anti-rat albumin rabbit antibody (Cappel, Cochranville, PA). Primary antibody was visualized by using labeled streptavidin biotin DAKO LSAB kit (DAKO, Carpinteria, CA).

Results

Cell attachment and formation of multilayer hepatocyte aggregates

Hepatocytes obtained from adult rats were attached to substrates and cultured in a HDM supplemented with or without 10% FCS or 0.03 to 3% BSA. When cultured on the surface of ePTFE in a FCS-supplemented medium, hepatocytes spread poorly, retaining their cuboidal shape (Figures 1a,c), and 4 to 5 days later reorganized themselves into multilayer cell aggregates (Figures 2a,c), as revealed by scanning electron microscopy.

The morphological characteristics of hepatocytes were influenced by the modification of the ePTFE surface as well as the culture medium. Hepatocytes cultured on a PVA-coated ePTFE surface in a FCS-

Figure 7. ELISA for albumin secreted per 10^5 hepatocytes cultured on various substrates. Hepatocytes were cultured for 28 days in a FCS-supplemented medium. ELISA was carried out as described in Materials and Methods. Each value is the mean $\pm$ SD of three experiments except for Ps (14, 21 and 28 days: single experiment).

supplemented medium formed many more multilayer cell aggregates than those cultured on an uncoated ePTFE surface (T. Tokiwa, unpubl.). Such highly multilayered cell aggregates were firmly attached to the PVA-coated ePTFE surface and not easily detached from the surface by pipetting. They were also noted when the cells were cultivated in a BSA-supplemented medium on PVA-coated ePTFE surfaces. The size of cell aggregates seemed to increase with increasing concentrations of BSA (Figures 3a,b,c).

In hematoxylin-eosin stained sections of the aggregates, the majority of cells looked normal, but there were also some degenerated cells including denucleated cells (Figures 4a,b,c).

These degenerated cells appeared even in the 3 day cell aggregates, but seemed to increase in number during further cultivations. On the other hand, when cultured in a FCS- or BSA-free medium, hepatocytes were attached, spread well (Figures 1b,d) and then formed cell monolayers on both PVA-coated (Figure 2b) and uncoated ePTFE surfaces as did the cells on a collagen-coated polystyrene surface (Figure 1f).

LDH levels

The value of LDH before culture (day 0) was 11.29 IU/l. After culture, regardless of the culture substrates, LDH values in the medium were unchanged through-

out the experiment except for those at day 2, suggesting that the degree of cell injury is very small in the present culture systems. Data were revealed as a percentage of day 0 value as shown in Figure 5. The LDH leakage at day 2 was due probably to cell injury at the inoculation procedure of hepatocytes. There was no difference between FCS-supplemented and -free culture medium regarding LDH leakage, although only the former data shown here.

DNA synthesis

In [3 H]thymidine incorporation study, when cultured on PVA-coated or uncoated ePTFE surfaces, hepatocytes exhibited remarkably lower DNA synthesis rate than those cultured on a collagen-coated polystyrene surface. The addition of FCS to the culture medium tended to lower the DNA synthesis rate (Figure 6).

Albumin production

The cells cultured on PVA-coated ePTFE surfaces in a FCS-supplemented medium were found to secrete large amounts of albumin (more than 4000 ng/ 10^5 cells/day) for at least 21 days as shown in Figure 7. In contrast to this, the cells cultured on uncoated ePTFE surfaces and a collagen-coated polystyrene surface in

Figure 8. Immunocytochemistry for albumin in the hepatocytes cultured on PVA-coated ePTFE surfaces. Hepatocytes were cultured for 9 days in a FCS-supplemented (a,b) or -free (c) medium. Immunocytochemistry was carried out as described in Materials and Methods. (a) positive staining, (b) primary antibody-minus control.

the same medium secreted albumin only for 14 days and 7 days, respectively.

Immunocytochemical studies using sections show that multilayer hepatocyte aggregates formed on a PVA-coated ePTFE surface in a FCS-supplemented

medium are positively stained as compared with a primary antibody-minus control and with the cells in a monolayer culture (Figures 8a,b,c).

Discussion

The ability of hepatocytes to organize into three-dimensional structures as spheroids or multilayer cell aggregates is hypothesized to contribute to their enhanced liver specific functions (T. Tokiwa, unpubl.). The formation of multilayer hepatocyte aggregates attached to the substrates have been noted by culturing hepatocytes on the surfaces coated with synthetic biocompatible polymers (Akaike *et al.*, 1993). The present report is the first one showing that multilayer aggregates of hepatocytes are formed and attached on porous polymer substrates.

The present results show that hepatocytes cultured on ePTFE surfaces, especially of the PVA-coated ePTFE, expressed higher level of albumin secretion and lower DNA synthesis capacity than those cultured on Ps. Hepatocytes have previously been reported to exhibit an inverse relationship between growth and differentiation *in vitro* (Ben-Ze'ev *et al.*, 1988). Present data suggest that the differentiation of rat hepatocytes could be improved by cell aggregation on a PVA-coated ePTFE surface.

Many studies show that hepatocyte spheroids or multilayer hepatocyte aggregates continue to secrete plasma proteins such as albumin and transferrin in long term culture, but the amounts of secreted proteins decrease with time in culture. The secretion of albumin by multilayer hepatocyte aggregates was maintained for up to 21 days in the present study. However, the amounts decreased with time in culture. Whether the decrease of differentiated functions by hepatocyte spheroids reflects cell necrosis or dedifferentiation has been discussed (Roberts and Soames, 1993; Tong *et al.*, 1994). The present results show that degenerated cells in the cell aggregates were visible at day 3 and seemed to increase in number in continuous cultures. Tong *et al.* (1994) report that the reduction of secreted proteins in human hepatocyte spheroids is not accompanied by expression of fetal proteins such as alpha-fetoprotein, suggesting that the decrease of amounts of albumin in the present culture systems may not be due to cell dedifferentiation, but to cell necrosis.

Koide *et al.* (1990) reported that the addition of FCS inhibited spheroid formation and suggested an importance of a serum-free culture in its formation. In the

present study, however, we show that multilayer hepatocyte aggregates are formed on PVA-coated ePTFE surfaces in the presence of FCS or BSA, suggesting that the results will address the use of ePTFE for an extracorporeal liver support since both hepatocytes and ePTFE will be exposed to blood or plasma in such a support.

The mechanism of multilayer hepatocyte aggregates formation on PVA-coated ePTFE surfaces in a FCS- or BSA-supplemented medium remains to be studied. It is well known that texture, porosity and other physical properties and surface charge, hydrophobicity and other chemical properties of the polymer substrates can influence cell behaviors such as cell adhesion and growth (Baldwin and Saltzman, 1996). It is also noted that limiting cell spreading on substrates may be important to maintain hepatic functions (Tong *et al.*, 1994). Although further experiments are needed, it is likely that proteins such as serum albumin are adsorbed to a PVA-coated ePTFE surface, and thereby limit cell spreading and affect subsequent cell differentiation or formation of multilayer cell aggregates.

Since ePTFE has such advantageous characteristics as it is chemically inert, highly insoluble and physically strong, the present results suggest that ePTFE is useful as a substrate for applications in tissue engineering such as for use in a hybrid-type artificial liver device.

In conclusion, functional hepatocyte cultures can be obtained in multilayer cell aggregates immobilized on a PVA-coated ePTFE surface in a FCS or BSA-supplemented medium.

Acknowledgements

This work was supported in part by the New Energy Industrial technology Development organization

(NEDO). The authors express special thanks to Xian-ying Meng and Xiao-dong Zhou for expert technical assistance.

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