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Original

Development of a New Bioartificial Liver Support System Using a Radial-flow Bioreactor

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Abstract: There is an increasing number of patients with severe liver disease that requires whole organ transplantation or living-related split liver transplantation. This has resulted in a shortage of donor organs, which is particularly problematic and still awaits resolution. Bioartificial liver (BAL) support systems have been developed with the aim of supporting patients with life-threatening liver disease until their liver recovers. Here, we describe a high performance three-dimensional rat hepatocyte culture system using a radial-flow bioreactor (RFB) with a polyvinyl alcohol (PVA) membrane as a small-scale BAL support system. Hepatocytes from male Sprague-Dawley rat livers were isolated and divided into two groups as follows. Group A: isolated hepatocytes were maintained in culture medium as controls; and group B: isolated hepatocytes were injected into the medium chamber of the RFB-PVA culture system. Sampling was carried out every 48 h to analyze the concentrations of ammonia and albumin in the medium. Light and electron microscopic examination of hepatocytes explanted from the PVA membrane was also performed. Albumin production and urea synthesis by cells in group B were both significantly higher than in group A. Hematoxylin-Eosin staining of the cells in group B showed that three-dimensional cell masses were attached to the PVA membrane. It also showed that the cells were stably proliferating in the porous spaces of the PVA. Scanning electron microscopic images of group B also showed clusters of hepatocytes attached to the PVA membrane. Hepatocyte clusters growing in the RFB-PVA culture system retained their biological function and were stable in the porous spaces of the PVA membrane. This cell culture system may be useful for the development of new BAL support systems.

Key words: bioartificial liver, radial-flow bioreactor, three-dimensional culture, polyvinyl-alcohol membrane.

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Introduction

Acute liver failure (ALF) is a significant health problem that is increasing in incidence in Japan. It is frequently characterized by rapid deterioration that leads to coma and death. The only effective therapy is liver transplantation. Increased numbers of patients with liver disease as well as expanding indications have led to an increase in the number of liver transplants. This has resulted in a chronic worldwide shortage of donor livers and an increase in both the waiting time and the mortality rate for prospective transplant recipients.

Liver function support strategies have been developed because of the scarcity of donor organs. These strategies aim to support patients with borderline functional liver cell mass either until an appropriate transplantable organ becomes available or until the liver recovers from injury[1–8]. Examples of non-biological systems that have been developed include plasma exchange[9–12], albumin dialysis[13–15], hemo (dia) filtration[16–18], and sorbent-based devices[16–25]. These systems are able to remove the toxins that accumulate because of hepatic failure, but their utility is limited by their inability to provide some liver-specific functions. In contrast, hepatocyte-based devices are able to provide whole liver function, including detoxification, biosynthesis, and biotransformation[5, 26, 27]. The development of clinically effective bioartificial liver (BAL) devices requires a high-density device that contains highly functional, viable cells.

The function of cultured hepatocytes can improve when they are grown as a three-dimensional (3D) culture rather than as a monolayer. The 3D culture conditions more closely resemble the normal tissue environment than those in culture dishes. We used a radial-flow bioreactor (RFB) to create 3D culture conditions. The RFB is a cylindrical culturing reactor filled with a solid matrix. The medium is pumped from the periphery toward the reactor center at an increasing rate, which is sufficient to supplement the cells with oxygen and nutrients. The matrix consists of a polyvinyl-alcohol (PVA) membrane with a high pore density that allows for a large surface area for cell attachment and the reduction of shear force caused by direct flow of the medium toward the cells[28–32].

In this study, we investigated the functional performance of a small-scale 3D culture system for hepatocytes using a RFB with a PVA membrane.

Materials and methods

Chemicals

Dulbecco's Modified Eagle medium (DMEM), Hepes, bovine serum albumin (BSA), galactose, L-proline, nicotinamide, ascorbic acid, dexamethasone, fetal bovine serum (FBS), trypsin-EDTA, calcium chloride, sodium alginate, and phosphate-buffered saline (PBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rat tail collagen type I was purchased from Collaborative Biomedical Product, (Bedford, MA, USA). Penicillin and streptomycin (PC / ST) were purchased from Omega Scientific (Tarzana, CA, USA). Recombinant human epidermal growth factor (rhEGF) was purchased from R&D (Minneapolis, MN, USA). Insulin-Transferrin-Selenium (ITS) was purchased from Gibco BRL (San Francisco, CA, USA). NaCl, KCl,

$NaHCO_3$, and NaH_2PO_4 were purchased from Wako Chemical Co (Tokyo, Japan).

Animals

This study was approved by the Committee on Animal Ethics in the Care and Use of Laboratory Animals of the Showa University Medical School. Male Sprague-Dawley rats (Saitama Experimental Animals Supply Co., Ltd, Saitama, Japan) weighing approximately 200–250 g were housed for more than 1 week with 12-h light / dark cycles prior to surgery. Animals were housed in a climate controlled (21℃) room with a 12-h light / dark cycle and were given tap water and standard laboratory rat chow *ad libitum*. All operations were performed under general anesthesia (ether) using a sterile surgical technique.

Hepatocyte isolation

Hepatocytes were harvested by a two-step EDTA / collagenase digestion *in situ*, as described previously[33]. Briefly, under general anesthesia and portal vein cannulation, in vivo perfusion of the liver was performed with an EDTA / collagenase solution at 37℃. The liver was excised and transferred to a culture dish containing DMEM supplemented with 10% FBS at 4℃. The capsule was peeled back from all lobes and the tissue was gently combed to isolate and suspend hepatocytes in the DMEM solution. Hepatocytes were filtered through mesh, suspended in DMEM solution and centrifuged at $50 \times g$ for 5 min. The cell viability as assessed by the trypan blue exclusion test was greater than 80% after enrichment through a Percoll density gradient.

Radial flow bioreactor

RFBs with either a 5 ml or 30 ml capacity as well as the RFB culture system were used in this study (ABLE Corporation, Tokyo, Japan)[32, 34, 35]. Porous PVA resin (Muromachi Kagaku, Tokyo, Japan) was used as the scaffold in the RFB (Fig. 1A). The inner space for the scaffold in the 5-ml RFB employed a central spiral coil with a circulation of 3 mm, and was filled with porous PVA resin. Isolated cells were injected into the medium chamber of the RFB culture system, which was filled with DMEM supplemented with 10% FBS (pH 7.6). The medium was circulated between the RFB and the medium chamber using a circulation pump (Fig. 1B). The cells remained attached to and grew on the scaffold while the medium was circulated. The concentration of ammonia was assayed using a urea nitrogen diagnostic kit (Biopharmigen) in samples of medium taken at 48 h intervals. The albumin concentration in the medium is measured using the Rat Albumin Enzyme Immunoassay Kit (Maassy Cedex).

Floating culture

A floating culture system was used to culture hepatocytes (SCHOTT Nippon K.K, Tokyo, Japan) using a 100 ml medium bottle and a magnetic stirrer spinning at a rate of 150–200 rpm (Fig. 1C). Hepatocytes were suspended in the culture medium, which allowed the 3D cell culture to proceed.

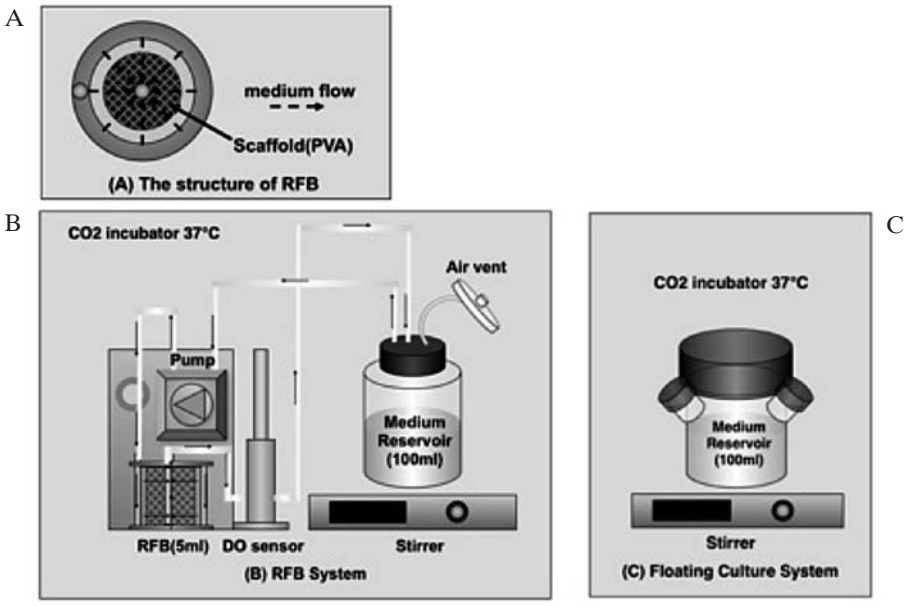

Fig. 1. Schematic illustrations of the two culture systems used
(A) Structure of the reactor : The polyvinyl-alcohol (PVA) scaffold is placed in the reactor as a support for seeding hepatocytes. Culture medium flows from the peripheral zone to the center inside the reactor. (B) The radial-flow bioreactor (RFB) system : RFBs with a reactor volume of 5 ml was used for the 3-D culture of hepatocytes in a CO_2 incubator. Dissolved oxygen and upstream static pressure were measured to monitor the condition of hepatocytes in the bioreactor. (C) Floating culture system : The medium reservoir has a volume of 100 ml and contains a magnetic stirrer bar inside the reservoir that spins at 150–200 rpm. Hepatocytes were suspended in the culture medium and were cultured in three dimensions.

Measurement of albumin secretion

Aliquots of culture medium were collected at 1, 3, 5, and 7 days of culture and the albumin concentration was assayed using a competitive enzyme linked immunosorbent assay (ELISA) using the Rat Albumin Enzyme Immunoassay Kit. Briefly, samples and the standard were seeded respectively into a 96-well microplate. Whereupon 0.05 ml of rat albumin AchE together with 0.05 ml rat albumin antiserum was added to each well. The plate was covered with plastic film and incubated for 16 h at 4°C. Ellman's Reagent (0.2 ml) was then added to the wells, which were incubated with shaking on an orbital shaker in the dark at room temperature for 20–30 min. The absorbance of each well was read between 405 nm and 414 nm (Wellreader SME 3400 of Iwaki Glass Co., Ltd, Shizuoka, Japan) and the albumin concentration was calculated.

Measurement of urea nitrogen synthesis

A final concentration of 2.0 mm ammonium chloride was added to the culture medium at 1, 3, 5, and 7 days. After incubation for 6 h, the urea nitrogen concentration was measured using a urea nitrogen diagnostic kit (Biopharmigen) and the absorbance was measured using a spectrophotometer (UV-1200, Shimadzu Co., Ltd, Kyoto, Japan) to determine urea synthesis.

Histological studies

Explanted hepatocytes from the PVA membrane were fixed in 10% formaldehyde for histological examination. Light microscopy was performed on paraffin-embedded sections stained with hematoxylin-eosin (H-E) and periodic acid-Schiff (PAS) stain.

Immunohistochemical staining of albumin

The hepatocytes were cultured on the PVA scaffold in the 5 ml RFB and the scaffolds were then removed from the reactor and fixed with 20% formalin neutral buffer solution (pH 7.4; Wako Chemicals, Osaka, Japan) at 4°C for 1 h. The cells on the scaffold were washed twice with PBS, and dehydrated by treatment with a series of solutions with increasing concentrations of ethanol. The dehydrated cell-scaffold material was embedded in resin using the Historesin Plus Embedding Kit (Leica, Heidelberg, Germany). Sections of 5 μm thickness were prepared and each section was stained with toluidine blue and observed under a microscope (BF-50; Olympus, Tokyo, Japan).

Ultrastructual examination

After removing the cultured hepatocytes, the ultrastructure of the PVA membrane was examined using scanning electron microscopy (SEM). The PVA membranes were treated for SEM analysis by fixation with 2% glutaraldehyde (pH 7.4) at 4°C for 24 h. The membranes were then washed and the post-fixed cells were dehydrated through a graded alcohol series, dried in hexamethyldisilazane and sputter coated with gold palladium and examined in a VE-7800 Keyence SEM.

Statistical analysis

Values are expressed as mean ± standard deviation. Statistical significance of the differences between the two groups was determined using Student's *t*-test.

Results

Hepatocyte function in the RFB and the floating culture system

This study cultured hepatocytes in either a 5 ml RFB using a PVA scaffold or a floating culture system and assessed the cellular function of the hepatocytes under these conditions by measuring the synthesis of albumin and urea. A time course of albumin concentration in the hepatocyte RFB-PVA culture system compared to the floating culture system showed significantly higher albumin production in the RFB-PVA culture system at all time points examined (Fig. 2). Urea synthesis by hepatocytes in the RFB-PVA culture system was also significantly higher than in the floating culture system at all time points examined (Fig. 3).

Morphological features

Thin sections of the scaffolds were prepared after hepatocyte culture and were studied microscopically. Representative photomicrographs of the sections show 3D aggregates of cells attached

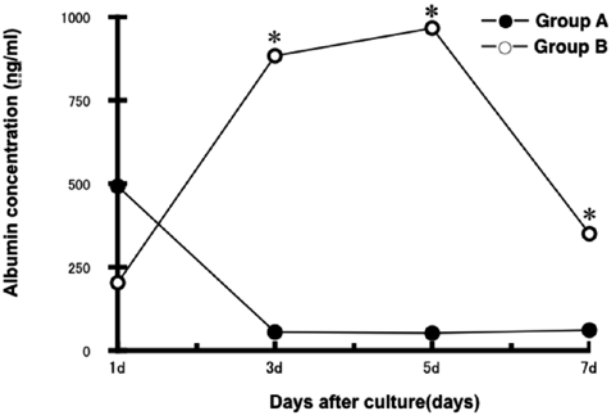

Fig. 2. Albumin production by cells cultured in the RFB and the floating culture system. Data are expressd as mean ± SD (n=3). Group A: floating culture system, Group B: RFB-PVA culture system. (*$P<0.05$ compared with Group A).

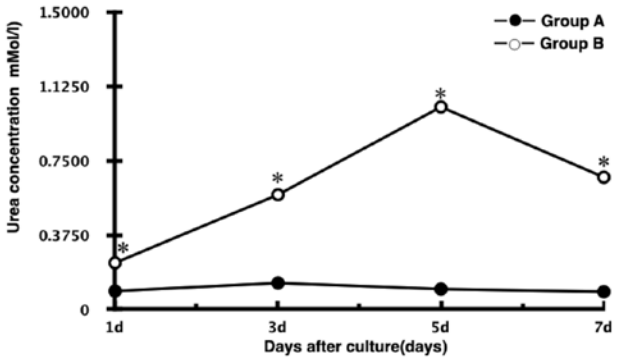

Fig. 3. Urea synthesis by cells cultured in the RFB and the floating culture system. Data are expressed as mean ± SD (n=3). Group A: floating culture system, Group B: RFB-PVA culture system. (*$P<0.05$ compared with Group A).

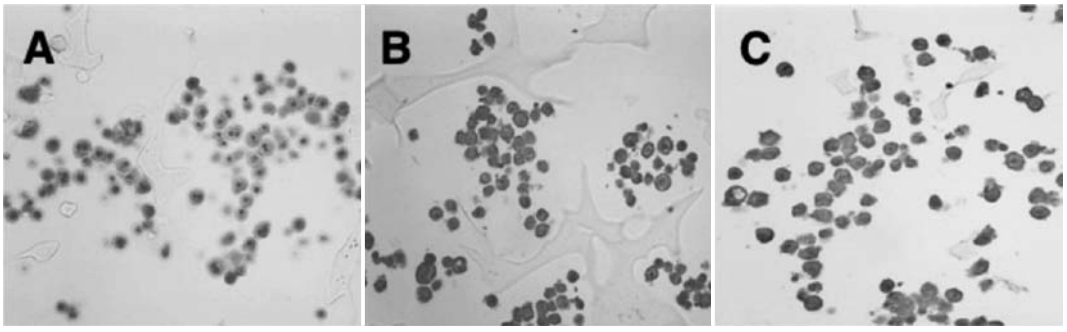

Fig. 4. Photomicrograph of hepatocytes cultivated on the RFB-PVA culture membrane
A: H-E stained sections of hepatocytes cultivated for 7 days on the PVA membrane (×200).
B: PAS stained sections of hepatocytes cultivated for 7 days on the PVA membrane (×200).
C: Albumin stained sections of hepatocytes cultivated for 7 days on the PVA membrane (×200).

to the PVA membrane, and demonstrate that the cells were proliferating stably in the porous spaces of the PVA (Fig. 4A). Immunohistochemical studies showed that hepatocytes cultured in the RFB-PVA culture system expressed PASs and Albumin at least 7 days after cultivation (Figs. 4B, C).

Electron microscopy

A representative SEM image shows the PVA membrane alone (Figs. 5A, B) and well-attached clustered hepatocytes on the PVA membrane (Figs. 5C, D). The hepatocytes cultured in the RFB-PVA culture system formed cell clusters that were 80–200 mm in diameter. These cell clusters grew within the pores of the PVA membrane over a period of 3 days (Fig. 5D) and were equally distributed throughout the scaffold (Fig. 5C).

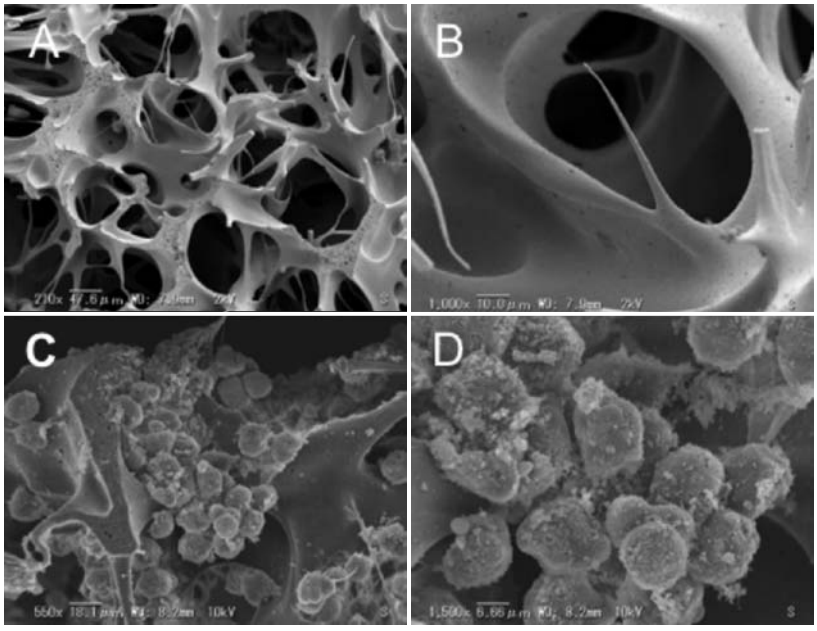

Fig. 5. Scanning electron microscopic image of cell clusters of rat hepatocytes growing on the PVA scaffold

A, B : Scanning electron microscopic image of the porous PVA scaffold at 3 days after cell seeding (bar, A : 47.6 μm, B : 10.0 μm).

C, D : Scanning electron microscopic image of cell clusters of rat hepatocytes growing on the PVA scaffold. Rat hepatocytes are attached to the pores of the PVA scaffold.

Scanning electron microscopic image of cell clusters of HepG2 cells formed on the PVA membrane at 3 days after seeding (bar, C : 18.1 μm, D : 6.66 μm).

Discussion

ALF is a severe disease that, despite recent therapeutic advances, remains associated with significant morbidity and mortality. Recently, liver transplantation has become a remarkably effective life-saving treatment for patients with fulminant hepatic failure and end-stage chronic liver disease[1–5]. Increases in both the number of liver transplantation procedures and the indications for the procedure have resulted in a chronic worldwide shortage of organs for transplantation. This is the situation despite alternative techniques being employed such as split-liver and living donor transplantation. As a result, waiting times and mortality for prospective transplant recipients have also increased.

One possible alternative to transplantation in some patients is the BAL support system, which can provide provisional hepatic function until a transplantable liver becomes available. In the present study, we tested the development of a small-scale BAL support system using a PVA membrane for the 3D culture of hepatocytes in a RFB. Primary rat hepatocytes actively survived and formed cell clusters in the porous scaffolds more efficiently than in a floating culture system. The cultured hepatocytes exhibited excellent cellular function as evidenced by the synthesis of albumin and urea in the RFB-PVA culture system. Hepatocytes cultivated for 7 days

in the RFB-PVA culture system secreted an eight-fold greater amount of albumin and a five-fold greater amount of urea than in the floating culture system.

Various types of bioreactors have been developed to support the growth of high-density 3D cell cultures. In particular, cultures using either microcarriers[41–44] or hollow-fiber modules[31, 45, 46] have been described.

In 2004, Demetriou *et al* reported the development of the HepatAssist® BAL device[26]. An early version of the HepatAssist® was the first BAL device tested in FDA-approved phase I / II / III clinical trials (Arbios Systems, Inc., Pasadena, CA, USA). The HepatAssist® BAL device incorporated collagen-coated dextran microcarriers that provided the hepatocytes with an attachment surface. RFBs are one type of bioreactor that can be used for high-density 3D cell culture. In the RFB, liquid medium flows convergently from the periphery to the center (Fig. 1A), enabling equally efficient gas and nutrient exchange in different parts of the scaffold. The use of RFBs can enhance the cellular function of the cultured cells[34, 35]. Hongo *et al* demonstrated that the cell density in the RFB was 4–15 fold higher than in microcarrier or hollow-fiber cultures[32].

In this study, we focused on using an RFB that enables the growth of a highly functional 3D culture. The performance of RFBs largely depends on the inclusion of a scaffold to provide a support matrix for cell attachment. Miyoshi *et al* found that fetal liver cells could be cultivated at high density on a polyvinyl foam (PVF) resin[47]. They were able to maintain a high-density cell culture (7×10^7 cells / cm^3) over 30 days of culture and the functioning of the cells was assayed by measuring the albumin production rate. They noted that after the first two weeks the rate of albumin production rate[47]. Kataoka *et al* developed a new porous organic inorganic hybrid scaffold consisting of tetraethoxysilane (TEOS) and polydimethylsiloxane (PDMS) and employed a sol–gel method with sieved sucrose particles that acted as a porogen[35]. This hybrid material was more suitable than PVA scaffolds to support the culture of the human HepG2 hepatocellular carcinoma cell line that was used in that study. We propose that primary hepatocytes should be used as a cell source rather than a transformed cell line such as HepG2 because cell lines are likely to have a reduced level of liver-specific function. In a preliminary study, we used two other types of scaffolds in the RFB, with porous beads composed of either silica or hydroxyapatite[32]. Primary hepatocytes could not attach to beads made from either of these materials (data not shown). Therefore, we selected a PVA membrane for as a solid support to culture primary hepatocytes in the RFB.

In this study, we observed the synthesis of albumin and urea by primary hepatocytes grown in the RFB-PVA culture system, which represents the appropriate functioning of these cells. Thin sections of the scaffolds were examined microscopically, with H-E sections showing that cells were stably attached to the 3D PVA membrane, and these cells were proliferating in the porous spaces of the PVA. Moreover, SEM images showed clustered hepatocytes attached to the PVA membrane. Therefore, we propose that hepatocytes and hepatocyte-derived cell lines exhibit improved specific cellular functions in cell clusters formed in the RFB with a PVA membrane than in other systems tested to date.

As our understanding of the pathophysiology of liver failure improves, we propose that the RFB system will continue to be developed. This system should provide : (i) detoxification ; (ii) replenishment of specific liver functions ; and (iii) stimulation of hepatic regeneration in combinations that are appropriate for the individual patient. The RFB-PVA BAL could be used to treat various forms of liver failure or patients with liver failure at various stages of illness. Further studies of this promising technology are required to assure that it can be scaled-up to provide a clinically-useful treatment option.

Conflict of interest disclosure

The authors have neither financial support nor relationships to disclose.

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