Biomaterials 35 (2014) 8983–8991

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Immune-protected xenogeneic bioartificial livers with liver-specific microarchitecture and hydrogel-encapsulated cells

Da Yoon No [a, 1], Gi Seok Jeong [a, 1], Sang-Hoon Lee [a, b, *]

[a] Department of Biomedical Engineering, Korea University, Seoul 136-701, Republic of Korea
[b] KU-KIST Graduate School of Converging of Sciences & Technologies, Korea University, Seoul 136-703, Republic of Korea

ARTICLE INFO

Article history:
Received 24 May 2014
Accepted 10 July 2014
Available online 1 August 2014

Keywords:
Hepatocyte
Tissue engineering
Bioartificial liver
Xenotransplantation
Microencapsulation
Hydrogel

ABSTRACT

Development of a xenogeneic biological liver support is important in providing a bridge to transplantation or liver regeneration, thus helping to overcome the chronic shortage of liver donors. Among the critical factors in developing biological liver support are the creation of *in vivo* mimetic micro liver tissue (mLT), especially mLTs containing liver-specific ultrastructure, and an encapsulation method that can package massive numbers of cells while providing immune-protection from the host immune system. We describe here the development of mLTs that include liver microarchitecture and their *in situ* encapsulation in hydrogel composites. Concave microwells and the tri-culture of three types of primary liver cells were applied for the construction of mLTs showing excellent liver functions and long-term (>1 month) viability *in vitro*. Large quantities of rat mLTs were encapsulated in collagen-alginate composites, implanted into hepatic failure mice and sustained their survival during regeneration of the remaining liver. The proposed liver support system offers xenogeneic hepatic assistance by mimicking native liver microarchitecture and providing immune-protection without the need for complicated devices or processes, and as such represents a promising system for recovery of organ function.

1. Introduction

Excessive alcohol consumption, viral hepatitis infection, misuse of medications, stress, and environmental factors contribute to a continuing increase in the number of patients with end-stage liver disease [1]. In the United States, liver disease is the twelfth-leading cause of death, accounting for 10.8 deaths per 100,000 population in 2011 [2]. Liver transplantation is the definitive treatment for liver failure, but only 10% of patients with fulminant hepatic failure undergo transplantation because of a shortage of donors [3], and one-third of patients with liver failure die awaiting a transplant [4,5]. If an adequate allograft is not immediately available, temporary hepatic support may maintain the patient until a suitable liver allograft becomes available or until the patient's own liver regenerates sufficiently to resume normal function [6]. However, because the liver performs complex functions regulated by equally complicated mechanisms, currently available non-biological extracorporeal liver support systems can provide only very short-term detoxification support, and do not cover the full range of liver functions, especially synthesis [7]. Thus, there is an urgent need for biological liver support systems that can fully restore liver function to act as a bridge to transplantation or liver regeneration [8].

Cell-based liver support systems would require obtaining a large quantity of cells from available sources and their preparation as highly functional units in a short period of time. However, proper preparation of these cells is time consuming, whereas patients with hepatic failure require immediate treatment to recover their rapid loss of liver function. Thus, long-term storable functional liver units that can be used on an emergency basis are needed for the development of biological liver supports. However, the development of functional liver units that maintain their function and viability *in vitro* for long periods of time, especially ready-to-use liver units essential for the rapid treatment of patients with liver failure, is challenging. Several liver micro-tissue models have shown improved function and survival time *in vitro*, but many of these models lack the heterotypic interactions and the complex architecture of native liver-like sinusoids [9,10], especially liver-specific differentiated structures [11–13]. Moreover, current models result in cells that survive for only a few days and with low-level liver

* Corresponding author. Department of Biomedical Engineering, College of Health Science, Korea University, Jeongneung 3-dong, Seongbuk-gu, Seoul 136-703, Republic of Korea. Tel.: +82 2 940 2881; fax: +82 2 921 6818.
E-mail address: dbiomed@korea.ac.kr (S.-H. Lee).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.biomaterials.2014.07.009
0142-9612/

function [9]. Readily usable liver sources require the long-term viability and functionality of liver micro-tissue *in vitro*. This may be accomplished by constructing three-dimensional (3D) organoids, consisting of diverse parenchymal and nonparenchymal cells that provide an *in vivo*-like microenvironment that mimics cell signaling, cell–cell interactions, and morphogenesis of normal liver tissue [14–17]. The critical factor in developing this type of 3D liver tissue, especially one that can maintain long-term liver function, is the construction of sinusoids, which play a key role in the efficient transport of nutrients and oxygen to all cells and in the elimination of toxic secretions [18]. However, engineering such complicated liver tissues including sinusoids has not yet been achieved.

An additional critical criterion for biological liver support is choosing proper and available cell sources. Most studies to date have used xenogeneic primary hepatocytes, because normal human cells are difficult to acquire, due to the lack of liver donors, and liver tumor cells cannot fully perform liver-specific functions [19,20]. The use of xenogeneic sources, however, requires their protection from the host immune system, for example by encapsulation, especially for implantation [21]. Encapsulation of cells with adequate amounts of extracellular matrix (ECM) and hydrogel can provide mechanical stability and help cells survive longer, in addition to providing immune-protection [22,23]. However, packing a massive number of these cells into a small space while providing adequate nutrition and oxygen to the cells, as well as the transport of cell secretions, is challenging. Moreover, encapsulation in a short period of time is critical.

To overcome these challenges and develop the *in vivo*-mimicking and uniformly sized and shaped micro liver tissues (mLTs), including a sinusoid-like structure, we employed the method of tri-culture of three kinds of cells including parenchymal cells and non-parenchymal cells. Through this method, developed mLTs were expected to form the liver ultrastructures spontaneously. To develop the immune-protected xenogeneic liver support system, we have tried to encapsulate these mLTs with a sufficiently large number of controllable cells in hydrogel, providing xenogeneic hepatic assistance without the need for complicated devices or processes. To prove the potential ability of this biological liver support system as a bridge to transplantation for liver failure, we employed the mouse 90% partial hepatectomy induced liver failure model and expected that encapsulated mLTs could support the recovery of the liver function in a liver failure induced mouse during regeneration.

2. Materials and methods

All experiments were performed in adherence to the National Institutes of Health guidelines for the use of experimental animals and followed protocols approved by the Institutional review board of the Korea University.

2.1. Isolation of liver cells and engineering of micro liver tissue

Hepatocytes were isolated from adult male Sprague–Dawley rats (DBL, Seoul, South Korea), weighing 250 g, using a two-step collagenase perfusion procedure, as described previously [24]. Hepatic stellate cells (HSCs) [25] and sinusoidal endothelial cells (SECs) [11] were obtained simultaneously by centrifugation of supernatants from hepatocyte washes, as described. Isolated HSCs were cultured and expanded in high-glucose DMEM containing 10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin, and cells were used after 5–7 days of culture in 75-cm^2 tissue culture flasks. SECs were cultured in complete endothelial growth medium (EBM2 basal medium plus EGM-2 bullet kit; Cambrex, Walkersville, MD, USA), and cells were used after 4–5 days of culture on collagen I-coated 6-well plates. All experiments were performed in accordance with the National Institutes of Health guidelines for the use of experimental animals and followed protocols approved by the Institutional review board of Korea University. mLTs of all four types were generated using PDMS-based concave micromolds with thin PDMS membranes [26,27]. Each concave micromold has 88 concave wells. Hepatocytes were seeded into these wells and cultured for a few days to allow mono-culture spheroids to form. Dual-culture spheroids were formed by seeding the wells with a 3:1 mixture of hepatocytes and HSCs or hepatocytes and SECs. Tri-culture spheroids were formed by seeding a 3:1:1 mixture of hepatocytes, HSCs, and SECs and culturing for several days (Fig. 1A). 2×10^4 of cells were seeded in each micromold.

2.2. Assembly of immune protected liver tissue

Engineered mLTs were retrieved from the concave micromolds by pipetting. Retrieved spheroids from nine micromolds were collected in an Eppendorf tube and re-seeded onto 700 μm diameter concave micromolds followed by sedimentation and washing. To encapsulate gathered mLTs, 200 μl collagen-alginate composite solution was added dropwise into each concave mold, completely filling it. A porous membrane (Spectra/Por 5 dialysis cellulose; Cole-Parmer, USA) was spread to uniformly cover the solution-filled concave wells. An equal volume of 100 mM $CaCl_2$ solution (Sigma) was pipetted onto the porous membrane, followed by incubation at 37 °C for 30 min [21,28]. The collagen-alginate sheet containing mLTs was carefully separated from the micro-concave mold (Fig. 1B).

2.3. Induction of acute liver failure in mice and implantation of mLT sheets

Eight-week-old male C57BL/6 mice weighing 20–25 g (DBL, Seoul, South Korea) underwent 90% hepatectomy to induce hepatic failure as described previously [29]. Immediately after hepatectomy, these mice were intraperitoneally implanted with six sheets of encapsulated tri-culture mLTs and maintained for 2–8 weeks (Fig. 1C). Ten mice were used for each group. All experiments were performed in accordance with the National Institutes of Health guidelines for the use of experimental animals and followed protocols approved by the Institutional review board of the Korea University.

2.4. Scanning electron microscopy (SEM)

mLTs formed in concave microwells were fixed with 2.5% glutaraldehyde in deionized water for 1 h and gently washed 3–5 times with deionized water. For secondary fixation, spheroids were immersed in 1% osmium tetroxide in deionized water for 1–2 h. Fixed mLTs were subsequently dehydrated with a graded ethanol series (25%, 50%, 75%, 95%, and 100%), immersed in tert-butyl alcohol three times for 30 min each at room temperature, and frozen at −70 °C. The mLTs were freeze-dried until the tert-butyl alcohol had evaporated, mounted onto a specimen stub with graphite paste, coated with palladium alloy, and observed under a scanning electron microscope (JEOL Ltd, Tokyo, Japan).

2.5. Cell viability

Cell viability was assessed by incubating spheroids with 50 mM calcein-AM and 25 mg/ml ethidium homodimer-1 (Molecular Probes, USA) in culture medium for 40 min at 37 °C, followed by imaging under a light microscope.

2.6. Transmission electron microscopy (TEM)

mLTs formed in concave PDMS microwells for 7 days were fixed in a solution containing glutaraldehyde. The samples were sliced and observed under a transmission electron microscope (JEOL, Tokyo, Japan) operated at 80 kV.

2.7. Fluorescein diacetate accumulation

mLTs were incubated with culture medium containing 2.5 μg/mL of fluorescein diacetate (Sigama) for 40 min in an incubator. After 40 min, the medium was replaced, and the mLTs were incubated in the absence of fluorescein diacetate for 40 min. Fluorescein distribution in the mLTs was observed using confocal microscopy (Olympus, Tokyo, Japan).

2.8. Immunofluorescence staining

Cells were fixed with 4% PFA for 30 min at 4 °C and incubated in 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 20 min at room temperature. After rinsing with 0.1% BSA in PBS, the cells were incubated with Block Ace (Dainippon Pharma, Tokyo, Japan) at 4 °C for 30 min and subsequently incubated overnight at 4 °C with rabbit antibodies against serum albumin (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and cytochrome P450 reductase (Abcam, Cambridge, UK), mouse antibodies against hepatic sinusoidal endothelial cells (Abcam, Cambridge, UK) and rat CD31 (Abcam, Cambridge, UK). The cells were rinsed with 0.1% BSA in PBS and incubated at 4°C for 90 min with Alexa Fluor 488-conjugated or Alexa Fluor 594-conjugated anti-rabbit or mouse IgG secondary antibodies, as appropriate. The cells were incubated with DAPI (4,6-diamidino-2-phenylindole) for 5 min at room temperature, and confocal microscopic images were obtained (Olympus, Tokyo, Japan).

2.9. Cryosectioning of mLTs

mLTs were fixed with 4% (w/v) paraformaldehyde (PFA) at 4 °C for 60 min, washed gently three times with PBS, and soaked overnight in 20% sucrose in PBS at 4 °C. Samples were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Tokyo, Japan) and kept as frozen blocks at −80 °C. Frozen sections were cut to a thickness of approximately 14 μm using a cryostat and placed on