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Construction of three-dimensional vascularized functional human liver tissue using a layer-by-layer cell coating technique

Kazuki Sasaki [a], Takami Akagi [b], Tadafumi Asaoka [a], Hidetoshi Eguchi [a], Yasunari Fukuda [a], Yoshifumi Iwagami [a], Daisaku Yamada [a], Takehiro Noda [a], Hiroshi Wada [a], Kunihito Gotoh [a], Koichi Kawamoto [a], Yuichiro Doki [a], Masaki Mori [a], Mitsuru Akashi [b, *]

[a] Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Japan
[b] Building Block Science Joint Research Chair, Graduate School of Frontier Biosciences, Osaka University, Japan

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ABSTRACT

The creation of artificial liver tissue is an active area of research due to the shortage of donors for liver transplantation. Here we investigated whether a simple and efficient cell coating technique developed in our laboratory could be used to generate functional vascularized liver tissue. This technique creates three-dimensional tissue by loading cells sterically onto other cells that have been coated with layer-by-layer (LbL) nanofilms of fibronectin and gelatin, two extracellular matrix proteins. We used this technique to construct homogenous, dense, well-vascularized liver tissue from cryopreserved human primary hepatocytes, human umbilical vein endothelial cells, and normal human dermal fibroblasts. Using LbL cell coating technique resulted in higher cellular function in terms of human albumin production ($P < 0.01$) and cytochrome P450 activity ($P < 0.01$) in vitro. Furthermore, after being transplanted subcutaneously into NOD/SCID mice, the vascularized liver tissue showed greater albumin production in the early stage than non-vascularized tissue or a hepatocyte suspension ($P < 0.01$). Histological examination demonstrated that compare to non-vascularized tissue, there were many less-morphologically changed and intact hepatocytes in the vascularized tissue. This cell coating technique would be applicable to the generation of vascularized functional liver tissue for regenerative medicine in the future.

1. Introduction

Liver transplantation is a well-established treatment for end-stage liver disease and acute liver failure. Japan enacted an Organ Transplant Law in October 1997 that was revised in July 2010. Since the revision, the number of transplants performed using brain-dead donors has increased five-fold. There are currently about 400 patients on waiting lists for liver transplantation from brain-dead donors in Japan. However, donated liver grafts are only transplanted into about 50 patients a year due to a shortage of available organ donors [1,2]. To resolve this critical shortage, several groups have studied alternatives to liver transplantation, such as cell transplantation and liver tissue engineering [3–7].

Tissue engineering is expected to be one of the temporary treatments available to patients who suffer from hepatic failure. However, the liver has a complex architecture and performs many functions, including glucose and lipid metabolism, detoxification, production of serum proteins, and secretion of bile. In terms of successful liver tissue regeneration, there are still several issues with respect to the most appropriate cell source, scaffold [8], and blood vessels. In particular, it is critical to develop techniques to create microenvironments that promote cell engraftment [3].

Vascularization is critical in tissue engineering [9], because blood vessels are needed to supply nutrients and oxygen the tissue and to transport waste products. A number of technologies for liver tissue vascularization have been reported, including a top-down approach that uses three-dimensional (3D) printing [10–12], a biodegradable scaffold [13,14], a hydrogel [15,16], a microfluidic model [17,18], decellularization [19], a bottom-up approach using cell sheets [20], organoid formation [4,7], and spheroid formation by co-culture with human umbilical vein endothelial cells

Abbreviations: 3D, three-dimensional; CPHs, cryopreserved human primary hepatocytes; CYP, cytochrome P450; hAlb, human albumin; HUVECs, human umbilical vein endothelial cells; LbL, layer-by-layer; NHDFs, normal human dermal fibroblasts; ECM, extracellular matrix.

* Corresponding author.
E-mail address: akashi@chem.eng.osaka-u.ac.jp (M. Akashi).

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(HUVECs) and hepatocytes [21,22]. However, none of these technologies have succeeded in generating homogenous, thick, and highly dense live tissue with abundant blood capillaries that is stable enough to be engrafted and clinically utilized as a graft or as a bridge to transplantation.

Recently, based on the concept of layer-by-layer (LbL) cell assembly [23], we developed a novel cell coating technique that serves as a simple, rapid, and efficient way to create in vitro 3D vascularized tissue [24–27]. The cell coating technique is used to form nanofilms that are composed of extracellular matrix (ECM) proteins, such as fibronectin (FN) and gelatin (G; a collagen derivative), on the surfaces of cells. LbL cell coating is based on the interactions between ECM proteins and the cell surface. Most cells express integrin α5β1 on the cell surface, and the RGD sequence in FN can bind to this integrin. FN also has a collagen-binding domain that can bind to G. When LbL coating is performed repeatedly, multiple layers of FN-G are formed on the cell surface. The ECM nanofilms thus function as molecular glue that promotes the binding of adjacent cells to each other. When normal human dermal fibroblasts (NHDFs) and HUVECs are coated with FN-G and combined, a 3D structure that has a dense vascular network can be formed [25,27]. Morphologically, the blood vessels that are constructed using the cell coating technique are similar to native vessels [28]. When these 3D vascularized constructs are transplanted subcutaneously into the backs of nude mice, the engrafted artificial vascular networks anastomose with the host vasculature [29]. In addition, our group have also successfully constructed other 3D tissue models, including 3D heart muscle [30], skeletal muscle [31], skin that has blood vessel and lymph-like capillary networks [32], cartilage-like tissue [33] and a liver tissue model using the HepG2 hepatocellular carcinoma cell line [34]. The 3D liver tissue model that used HepG2 cells showed higher albumin production and cytochrome P450 (CYP) activity than 2D cultures [34].

We hypothesized that it would be possible to construct vascularized functional liver tissue by adding hepatocytes to a co-culture of HUVECs and NHDFs. Towards this end, we aimed to create a vascularized liver tissue model using a small number of cryopreserved human primary hepatocytes (CPHs) and our LbL technique and then to evaluate whether the vascularized tissue could be engrafted in NOD/SCID mice. The purpose of this study was thus to demonstrate that our original LbL cell coating technique could be used as a tool to generate vascularized and highly functional 3D liver tissue.

2. Materials and methods

2.1. Materials

All chemical reagents were used without further purification. FN from human plasma, bovine serum albumin (BSA), and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM), G, 10% formalin solution, and 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The following antibodies were purchased from Abcam (Cambridge, UK): monoclonal mouse anti-cytokeratin 18 antibody (CK18) (ab668, 1:500), polyclonal rabbit anti-human CD31 antibody (CD31) (ab28364, 1:50), monoclonal rabbit anti-human CD31 antibody (hCD31) (ab76533, 1:250), monoclonal mouse anti-human vimentin (hVim) (ab8069, 1:500), and monoclonal mouse anti MRP-2 antibody (MRP2) (ab3373, 1:200). The following antibodies were purchased from Dako (Glostrup, Denmark): polyclonal rabbit anti-human albumin (hAlb) (A0001, 1:2000), monoclonal mouse anti-human CD31 antibody (mhCD31) (IR610, 1:50), monoclonal mouse anti-human von Willebrand factor (hvWF) (M 0616, 1:50), monoclonal mouse anti-human CD34 antibody (hCD34) (M7165, 1:50), monoclonal mouse anti-human smooth muscle actin (αSMA) (M0851, 1:100), 3,3'-diaminobenzidine tetrahydrochloride, and EnVision™. Mayer's hematoxylin was purchased from MUTO PURE CHEMICALS Co., Ltd (Tokyo, Japan). The following were purchased from Life Technologies (Carlsbad, CA, USA): goat anti-mouse Alexa Fluor 488/546/647-conjugated IgG (1:200), goat anti-rabbit Alexa Fluor 488/546-conjugated IgG (1:200), 4′6-diamidino-2-phenylindole (DAPI), dihydrochloride (1:1000), fetal bovine serum (FBS), CellTracker Green CMFDA, CellTracker Red CMPTX, and ProLong gold antifade mounting medium. The cell culture inserts with 0.4-μm pore polyester membrane (Cat. No. 3470) and 3-μm pore polycarbonate membranes (Cat. No. 3414) were purchased from Corning (Corning, NY, USA). HUVECs and endothelial growth medium (EGM-2 MV) were purchased from Lonza (Allendale, NJ, USA). HepG2 cells were purchased from Cellular Engineering Technologies (Coralville, IA, USA). CPHs (non-plateable cryohepatocytes), thawing medium (Leibovitz 15 medium with Glutamax), and seeding medium (Williams E Medium with Glutamax reconstituted with 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 4 μg/ml of bovine insulin, and 10% v/v of FCS) were purchased from BIOPREDIC (Rennes, France).

2.2. Study design

First, based on our previous study that used the HepG2 cell line, we examined the efficacy of vascularization in liver tissue using HepG2 cells, determined the optimal ratio of HepG2 cells, HUVECs, and NHDFs, and compared the structure and vascularization of the tissue formed with and without the use of the LbL cell coating technique (Fig. 2). Second, we applied these results to CPHs (Fig. 4) and investigated the function of the liver tissue model in terms of protein synthesis and metabolism with and without cell coating and with and without vascularization. Third, we evaluated the effects of prevascularization of the liver tissue model by transplanting the tissue subcutaneously into the backs of NOD/SCID mice.

2.3. Construction of vascularized liver tissue using a cell coating technique

2.3.1. Preparation of the cells

The experiments used CPHs (or HepG2 cells), NHDFs (up to passage 8), and HUVECs (up to passage 8). One vial of CPHs was thawed in thawing medium and suspended in seeding medium according to the manufacturer's protocol. Thawed CPHs (viability > 80%) were used directly for tissue construction after LbL FN-G cell coating.

HepG2 cells and NHDFs were cultured in DMEM with 10% FBS. HUVECs were cultured in EGM-2MV. HepG2 cells, NHDFs, and HUVECs that were 80%–90% confluent in a 10-cm dish were treated with trypsin and EDTA and centrifuged at 160 × *g*. After the supernatant was removed, the cells were suspended in PBS and used in the following procedure.

2.3.2. Construction of liver tissue using cells subjected to the LbL FN-G cell coating treatment

A schematic showing the LbL FN-G cell coating technique and tissue production procedure is shown in Fig. 1A. As reported previously [30], CPHs and NHDFs were coated with FN and G using a filter-based LbL procedure. HUVECs were used without FN-G coating. A shaking incubator (SI-300, AS ONE, Japan) was used for the coating step. Briefly, FN in PBS, PBS, and G in PBS were added to a 6-well cell culture dish. After CPHs (viability > 75%) or HepG2 cells (viability > 95%) were immersed in a FN solution on a culture