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Basic Study

Syngeneic implantation of mouse hepatic progenitor cell-derived three-dimensional liver tissue with dense collagen fibrils

Hepatic progenitor cell–derived 3-D liver tissue model

Abstract

BACKGROUND

Liver transplantation is a therapy for irreversible liver failure; however, at present, donor organs are in short supply. Cell transplantation therapy for liver failure is still at the developmental stage and is critically limited by a shortage of human primary hepatocytes.

AIM

For hepatocyte implantation, we attempted to enable the implantation of extracellular matrices containing organoids consisting of hepatocytes and non-parenchymal cells.

METHODS

In vitro liver organoid tissue has been generated by accumulating collagen fibrils, fibroblasts, and hepatic progenitor cells on a mesh of polylactic acid fabric using a bioreactor; this was subsequently implanted into syngeneic wild type mice.

RESULTS

The *in vitro* liver organoid tissues generated transplantable tissues in the condensed collagen fibril matrix and were obtained from the mouse through partial hepatectomy.

CONCLUSION

Liver organoid tissue was produced from expanded hepatic progenitor cells using an originally designed bioreactor system. This tissue was comparable to liver lobules, and with fibroblasts embedded in the network collagen fibrils of this artificial tissue, it is useful for reconstructing the hepatic interstitial structure.

Key Words: Liver; 3-D tissue culture; Hepatic progenitor cells; Angiogenesis; Biomimetic extracellular matrix

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Core Tip: Liver transplantation is a therapeutic procedure used to recover liver function in patients with irreversible liver failure; however, presently there is a shortage of transplant organs available. Hepatic stem and progenitor cells are expected to allow regenerative medicine to produce a cell source as an alternative to whole organs. The portal branch-ligated, hepatic lobe-derived hepatic progenitor cells multiplied in a bioreactor chamber to form liver organoid tissues comparable to liver lobules. These organoid tissues were implanted into syngeneic mice. This PBL-derived hepatic progenitor cell line has the potential to proliferate, mature, and form implantable hepatic tissue.

INTRODUCTION

Liver transplantation is a therapy for irreversible liver failure; however, donor organs are in short supply at present[1, 2]. Cell transplantation therapy for liver failure is still at the developmental stage and has a critical problem in terms of a shortage of human primary hepatocytes [3]. Human embryonic stem/induced pluripotent stem (ES/iPS)

cell–derived hepatocytes are thought to be an alternative to human primary hepatocytes, but ES/iPS cells are difficult to differentiate into mature hepatocytes in culture [4, 5]. ES/iPS cell–derived immature hepatocytes have successfully developed into mature liver tissue in animals after their implantation [6, 7]. However, this process requires a great deal of time, effort, and expense in order to obtain a sufficient number of ES/iPS cell–derived hepatocytes in culture to achieve the amount needed for them to continue to proliferate. There have been several reports of rat hepatic progenitor cells, such as small hepatocytes [8] and Lgr5+ rat and mouse liver stem cells [9, 10] becoming established in culture. These hepatic stem/progenitor cells have the ability to proliferate and differentiate into hepatocytes and cholangiocytes [11, 12]. Recently, we also succeeded in establishing hepatic progenitor cell lines prepared from the portal branch-ligated hepatic lobe in mice (PBL-hepatic progenitor cell) [13]. These cells could differentiate into mature hepatocytes in the presence of oncostatin M, or to cholangiocytes in EHS gel.

Besides dissociated hepatocyte implantation, regenerative medicine is also expected to enable the implantation of extracellular matrices containing aggregate, or organoids consisting of hepatocytes and non-parenchymal cells [14-16]. *In vitro* liver organoid tissue has previously been generated by accumulating collagen fibrils, human fibroblast cell line (HFO cell), and human hepatocarcinoma cell line (Hep G2) on a mesh of polylactic acid fabric using a bioreactor [14]. Also, instead of HFO and HepG2, mouse embryonic fibroblasts and primary hepatocytes were used for this *in vitro* liver organoid tissue. These *in vitro* liver organoid tissues generated transplantable liver organoid tissues in the right portal vein branch-ligated *nu/nu* mouse with a condensed collagen fibril matrix [14]. The fibroblasts are embedded in the network collagen fibrils of this artificial tissue, and it is therefore useful for reconstructing the hepatic interstitial structure.

In this study, the PBL-hepatic progenitor cells were expanded and formed liver organoid tissue which was comparable to liver lobules using an originally designed bioreactor system, and was also implanted into its syngeneic wild-type mouse.

MATERIALS AND METHODS

Animals

Pregnant BALB/cA mice at 13.5 days post coitus (CLEA Japan, Tokyo, Japan) were used for embryonic fibroblast isolation. Six-week-old female and male BALB/cA Jcl and BALB/cA Jcl-nu/nu 3 mice (CLEA Japan, Tokyo, Japan) were used as transplant recipients. The animal protocol was approved by the Animal Experimentation Committee of the Tokyo Institute of Technology.

Cells and cultures

The PBL-hepatic progenitor cells were established in a previous study with portal vein ligated methods [13]. The cells were cultured in Williams' E medium (GIBCO Laboratories, Grand Island, NY) supplemented with 5% fetal bovine serum, 10 mmol/L nicotinamide (Sigma–Aldrich, St. Louis, MO), 0.1 μM dexamethasone (Sigma–Aldrich), 1×Insulin–Transferrin–Sodium Selenite Supplement (Roche Diagnostics, Mannheim, Germany), and 20 ng/mL Recombinant Mouse Epidermal Growth Factor (R&D Systems, Minneapolis, MN) in 5% CO_2 at 37 °C. These cells were passaged by treatment with 0.05% trypsin (Invitrogen) and 20 μM ethylenediaminetetraacetic acid (EDTA; NACALAI TESQUE, Kyoto, Japan).

Preparation of murine embryonic fibroblasts

A pregnant female BALB/c at 13.5 dpc (days post-coitum) was sacrificed by cervical dislocation, and embryos were removed. The limbs of the embryos were minced and treated with 0.25% trypsin (Invitrogen, Tokyo, Japan) + 1 mmol/L EDTA (about 2 mL per embryo) and incubated with gentle stirring at 37 °C for 10–15 min. The cells were subsequently cultured in DMEM containing 10% (v/v) FBS.

Generation of 3-D liver tissue culture model

As shown in Figure 1, the 3-D liver tissue culture model was generated by accumulating collagen fibrils, primary murine embryonic fibroblasts, and PBL-hepatic progenitor cells using a closed-loop system with a bioreactor chamber (diameter 17 mm; thickness 20

mm) developed by our group as previously reported [14, 17]. Briefly, primary embryonic fibroblasts (1.0×10^5 cells/mL) in 10% FBS and 7.5 mg/mL type I collagen prepared from calfskin (Koken Collagen, Tokyo, Japan) in Williams' E medium flowed through the closed-loop system at a predetermined flow rate (1-5 mL/min) for 6 h. Subsequently, the same medium was circulated through the closed-loop system, and PBL-hepatic progenitor cells (5.0×10^6 cells/mL) were injected using a syringe into the system upstream of the bioreactor chamber for 2 h. Finally, a suspension of fibroblasts (1.0×10^5 cells/mL) was circulated for 6 h.

Morphological analyses

The 3-D liver tissue culture models were fixed with Zamboni's fixative for light microscopy. The samples were dehydrated with an ethanol series and embedded in paraffin. The sections were stained with hematoxylin and eosin or AZAN and examined with a light microscope.

Hepatic function assay in liver tissue culture model

Urea production was quantified using a urea assay kit (Bioassay Systems, Hayward, CA, U.S.A.) in the medium 24 h after the addition of 2 mmol/L NH_4Cl. Albumin production was quantified in the medium by using an albumin EIA (Albuwell M) mouse kit (Exocell, Philadelphia, PA).

The metabolites of testosterone were quantified in the medium by HPLC analysis [18]. The 3-D liver tissue culture models were incubated with fresh medium containing 0.25 mmol/L testosterone and the medium was collected at 24 h. After sample treatment, HPLC analysis was performed using LC-10ADVP (Shimadzu, Kyoto, Japan) with Cadenza columns (Cadenza CD-C18) (Imtakt, Kyoto, Japan) and SPD-10A VP (Shimadzu, Kyoto, Japan).

Transplantation of liver tissue culture model

Under isoflurane anesthesia, mice were subjected to an upper-abdominal incision, followed by exposure and ligation of the left portal vein branch and subsequent hepatectomy of the left and middle lobes (70%). The 3-D liver tissue culture model was transplanted into the subcutaneous layer of a mouse. Two weeks later, the 3-D liver tissue culture model was removed for histological analysis of the vascular network.

Statistical evaluation

Results of multiple experiments were reported as the mean ± SEM. Statistical comparisons were made using a Tukey–Kramer method and a Welch *t*-test using the IBM SPSS Statistic 27.

RESULTS

Preparation of three-dimensional hepatic progenitor cell–derived 3-D liver tissue culture model

Type I collagen solution was circulated through a sheet of polylactic acid (PLA) into a reverse radial flow-type bioreactor, followed by the suspension of 5.0×10^6 cells of MEFs. In the next step, the suspension of 1.0×10^7 PBL-hepatic progenitor cells was circulated without oncostatin M. After these steps, the type I collagen solution was circulated again followed by the suspension of 5.0×10^6 MEFs. Finally a 3-D aggregate was prepared (Figure 1A). The surface of this 3-D aggregate made of PBL-hepatic progenitor cells, primary fibroblasts, and type I collagen was glossy and measured 17 mm in diameter and 1.5 mm in height. This glossy aggregate consisted of a layer of PBL-hepatic progenitor cells, sandwiched between two layers of collagen fibrils with MEFs, and was constructed on a sheet of PLA as shown in Figure 1G. Cross-sectional profiles of the 3-D liver tissue culture models were stained with hematoxylin-eosin and AZAN as shown in Figure 1D-F. The collagen layers were composed of densely packed collagen fibrils running parallel to the plane of the PLA sheet. A layer of PBL-hepatic progenitor cells 200-300 µm thick (Figure 1D), and two layers of collagen fibrils populated with embryonic fibroblasts, approximately 400 µm thick, were observed in

the 3-D liver tissue culture model. To culture the 3-D liver tissue model, the cylinder inside the bioreactor was changed from construction to culture (Figure 1B). The 3-D liver tissue culture model was cultured using a differentiation medium containing 20 ng/mL oncostatin M in the bioreactor for 12 days. After this, PBL hepatic progenitor cells differentiated into mature hepatocyte-like cells, in binuclear populations, and with a bile duct–like structure (Figure 1E). Collagen layers were maintained by the collagen fibers and fibroblasts on day 12.

Expression of liver-related genes in hepatic progenitor cell–derived 3-D liver tissue culture model

The expression of liver-specific genes was explored for the 3-D liver tissue culture model. To investigate whether the 3-D liver tissue culture models were able to show hepatic lineage differentiation (i.e., to mature into hepatocytes and bile duct cells), the cells were cultured in the presence of OSM. By day 12 of the culture in the hepatic lineage differentiation medium, qPCR analysis revealed that the cells expressed hepatocyte differentiation markers including: *Afp (a-fetoprotein); Albumin*; *Tat (tyrosine aminotransferase); Tdo (tryptophan 2,3-dioxygenase); Cps1 (carbamoyl-phosphate synthetase 1); Cyp1a2 (Cytochrome P450, family 1, sub-family a2); Cyp2e1 (Cytochrome P450, family 2, sub-family e1);* and *Abcc2 [ATP-binding cassette, sub-family C (CFTR/MRP), member 2]* (Figure 2A-H). On the other hand, the gene expression of *Bsep (bile salt export pump)* and ABCB11 (*ATP-binding cassette, sub-family B member 11)* decreased in the 3-D liver tissue culture model (Figure 2I). The expression of *CK19*, a representative marker for hepatic progenitor cell, was confirmed in the PBL-hepatic progenitor. The gene expression of *CK19* decreased in the 3-D liver tissue culture models (Figure 2J). In addition, PBL-hepatic progenitor cells expressed the *CD44* gene, a progenitor cell marker, which decreased in the 3-D liver tissue culture models (Figure 2K). After that, *CD44* expression increased in the 3-D liver tissue culture models on day 12 of culture (Figure 2K).

The 3-D liver tissue culture model exhibits expression of multiple liver-specific functions

The expression of several liver-specific functions, such as the production of urea and albumin and drug metabolism, were analyzed in the 3-D liver tissue culture models. The urea production was examined in cultured 3-D liver tissue culture models in a hepatic lineage differentiation medium containing 2 mmol/L NH_4^+. The level of urea production in the 3-D liver tissue culture model gradually increased and was significantly higher on days 6 and 12 than in the 2-D culture (Figure 3A). The amount of albumin released from the 3-D liver tissue culture model into the medium was measured in each medium on day 1, day 6, and day 12 by enzyme-linked immunosorbent assay. As seen in Figure 3B, the albumin level increased gradually from day 1 to day 12. These results suggest that the 3-D liver tissue culture model with PBL-hepatic progenitor cells had been differentiated.

To quantify P450 activities, the hydroxylated pattern of testosterone by cultured 3-D liver tissue culture models in a hepatic lineage differentiation medium, containing 250 μM testosterone, was examined using high-performance liquid chromatography. The concentration of each hydroxylated testosterone (6β-OHT, 7α-OHT, 16α-OHT, and 16β-OHT, respectively corresponding to oxidation by Cyp3a, Cyp2a4/5 and 2d9, Cyp2d9 and 2b, and Cyp2c29 and 2e) was quantified. The concentrations of hydroxylated testosterones, such as 6β-OHT, 7α-OHT, 16α-OHT, and 16β-OHT, in the media of the 3-D liver tissue culture models after a 12-day culture were measured. As compared with the hydroxylation levels of 6b-OHT, 7a-OHT, and 16a-OHT of the 2-D culture, the hydroxylation levels were significantly increased in the 3-D liver tissue models (Figure 3C).

The 3-D liver tissue was engrafted in partially hepatectomized mouse

The 3-D liver tissue culture model of PBL-hepatic progenitor cells was syngenically transplanted into the subcutaneous layer of a BALB/cA mouse which had received a 70% partial hepatectomy as shown in Figure 4A. The graft could be observed in the

subcutaneous layer two weeks after the transplantation. Microvascular networks run throughout this engrafted tissue. As can be seen in Figure 4B and C, the hematoxylin-eosin staining of this specimen in the graft area shows that collagen remained rich in the graft, fibroblasts existed in the collagen area, and vessel-like tube formation could be observed. To investigate whether the cells in these areas were hepatocytes, an immunohistochemical examination using staining with anti-albumin antibodies was carried out in Figure 4D. This confirmed that the PBL-hepatic progenitor cells were accepted as albumin-positive cells after transplantation. These results indicate that the 3-D liver tissue culture model was successfully grafted with angiogenesis in the partially hepatectomized mouse.

DISCUSSION

It is crucial to develop a technology that enables transplantable engineered tissues to be functionally engrafted and long-lasting, in order to maximize the therapeutic effects of this procedure [19-21]. There are reports of transplanted hepatocytes at several different extrahepatic sites such as the small intestine[22, 23]. Tissue engineering has been a promising procedure for providing transplantable tissues mimicking liver *ex vivo* [24, 25]. The attachment of hepatocytes to extracellular matrix scaffolds can help in their engraftment in extrahepatic sites [26, 27]. It was important to provide scaffold materials for hepatocytes to enable significantly greater hepatocyte survival in heterotypic transplantation [28]. The liver is encased mainly with collagen fibrils. By fabricating graded structures specific for target tissues and organs, one can obtain suitable scaffolds for tissue regeneration[29]. Taking into account the architecture of the liver, we have generated a 3-D liver tissue culture model of hepatic progenitor cells with a collagen fibril matrix using a bioreactor. Furthermore, defining and validating new sources is mandatory for ensuring functional hepatic cell supply [30]. Hepatic stem/progenitor cells have many advantages compared to adult hepatocytes as they are bipotent cells, so they can differentiate into hepatocytes and [20]cholangiocytes. Moreover, they have a high proliferation ability.

In this study, hepatic progenitor cells—PBL-hepatic progenitor cells—were used in an original procedure to generate a 3-D liver tissue culture model. The histological structure of this model resembled that of the liver, with respect to its capillary network and surrounding cell clusters. Because the PBL-hepatic progenitor cells have the potential to reproduce themselves, it is easy to prepare the necessary numbers of cells (1×10^7 cells order). The PBL-hepatic progenitor cell–derived 3-D liver tissue culture model reconstructed in a bioreactor produced cells that differentiated into hepatic-like cells, binuclear populations, and bile duct–like structures (Figure 1F). These cells expressed hepatocyte differentiation markers (Figure 2) after 12 days of culture. The PBL-hepatic progenitor cells differentiated not only into hepatic cells but also bile duct–like cells in a reconstituted collagen fibril matrix. In the 3-D liver tissue culture model derived from PBL-hepatic progenitor cells, the levels of urea and albumin production were gradually enhanced and significantly higher than those cultured in dishes on days 6 and 12 (Figure 3A and B). Cyp3a, 2a4/5, 2d9, and 2b activities were significantly increased in the 3-D liver tissue culture models (Figure 3C). These results suggest that the PBL-hepatic progenitor cells differentiated to matured hepatocytes in the 3-D liver tissue culture model.

Since PBL-hepatic progenitor cells differentiated into cells expressing hepatic functions in a 3-D liver tissue culture model, further investigation was carried out to determine whether PBL-hepatic progenitor cells also maintained their acquired functions after transplantation. After the implantation of the PBL-hepatic progenitor cell–derived 3-D liver tissue culture model into mice, the 3-D liver tissue culture model was grafted, and vessel-like tube formation could be observed (Figure 4B and C).

Also, the 3-D liver tissue culture model was grafted in the healthy mouse which was not received partial hepatectomy, and vascularization could be observed (Supplemental Figure 1A and B). On the other hand, the Matrigel-embedded PBL-hepatic progenitor cells were transplanted into male healthy mice or nu/nu mice, and vascularization could not be observed (Supplemental Figure 1C, D and E, F, respectively). PBL-hepatic progenitor cells differentiated into albumin positive cells (Figure 4D). This haptic tissue

consisted of fibroblasts and hepatic progenitor cells which can be differentiated to only hepatocytes and bile duct cells, not immune cells. Because some blood vessels could be observed after the implantation, lymphocytes and monocytes might be circulating. However, we could not detect Kupfer cells in the grafts. The 3-D liver tissue culture model was investigated for efficiency of transplantation in extrahepatic sites. These findings demonstrate that a reconstituted collagen fibril matrix can provide an extracellular microenvironment promoting the maturation of progenitor cells into hepatic cells. A local vascular network would allow nutrient and gas transport to the grafts. These findings could make a significant contribution to the liver graft shortage problem.

CONCLUSION

In conclusion, a 3-D liver tissue culture model was developed by using hepatic progenitor cells. The advantage of our system is that it consists of proliferative hepatic progenitor cells. The 3-D liver tissue culture models can be generated in an originally designed bioreactor within 24 h. By mimicking the structure of the natural liver, our system was effective in constructing a functional liver tissue model.

ARTICLE HIGHLIGHTS

Research background

Liver transplantation is a therapeutic procedure to recover liver function in patients with irreversible liver failure; however, there is a shortage of transplant organs available at present, which limits the availability of this treatment.

Research motivation

Portal branch-ligated hepatic progenitor cells are expected to allow regenerative medicine to produce a cell source to provide an alternate source for transplantation.

Research objectives

We aimed to development a liver model using hepatic progenitor cells.

Research methods

Hepatic stem/progenitor cells have the ability to multiply *ex vivo* and differentiate into hepatocytes and cholangiocytes. We have previously established hepatic progenitor cell lines derived from the hepatic tissues of mice after ligation of venous drainage. In this study, the portal branch-ligated hepatic lobe (PBL)–derived hepatic progenitor cells multiplied in a bioreactor chamber to form liver organoid tissues comparable to liver lobules. These organoid tissues were implanted into syngeneic wild-type mice.

Research results

In the 3-D liver tissue culture model, PBL hepatic progenitor cells differentiated into mature hepatocyte-like cells, in binuclear populations, and with a bile duct–like structure. And qPCR analysis revealed that the cells expressed hepatocyte differentiation markers. In the 3-D liver tissue culture model derived from PBL-hepatic progenitor cells, the levels of urea and albumin production and activities of Cytochromes P450 were gradually enhanced.

Research conclusions

By mimicking the structure of the natural liver, our system was effective for the construction of a functional liver tissue model.

Research perspectives

This PBL-derived hepatic progenitor cell line has the potential to proliferate, mature, and form implantable hepatic tissue.

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