Biomaterials xxx (2014) 1–11

Contents lists available at ScienceDirect

Biomaterials

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

Cryo-chemical decellularization of the whole liver for mesenchymal stem cells-based functional hepatic tissue engineering

Wei-Cheng Jiang [a,b], Yu-Hao Cheng [c], Meng-Hua Yen [b], Yin Chang [a], Vincent W. Yang [e], Oscar K. Lee [d,f,*]

[a] Institute of Biomedical Engineering, National Yang-Ming University, Taiwan
[b] Stem Cell Research Center, National Yang-Ming University, Taiwan
[c] Faculty of Medicine, National Yang-Ming University, Taiwan
[d] Department of Medical Research & Education, Taipei Veterans General Hospital, Taiwan
[e] Department of Medicine, Stony Brook University School of Medicine, Stony Brook, NY, USA
[f] Institute of Clinical Medicine, National Yang-Ming University, Taiwan

ARTICLE INFO

Article history:
Received 18 November 2013
Accepted 8 January 2014
Available online xxx

Keywords:
Acellular liver scaffolds
Mesenchymal stem cells
Hepatic-like tissues
Transplantation

ABSTRACT

Liver transplantation is the ultimate treatment for severe hepatic failure to date. However, the limited supply of donor organs has severely hampered this treatment. So far, great potentials of using mesenchymal stem cells (MSCs) to replenish the hepatic cell population have been shown; nevertheless, there still is a lack of an optimal three-dimensional scaffold for generation of well-transplantable hepatic tissues. In this study, we utilized a cryo-chemical decellularization method which combines physical and chemical approach to generate acellular liver scaffolds (ALS) from the whole liver. The produced ALS provides a biomimetic three-dimensional environment to support hepatic differentiation of MSCs, evidenced by expression of hepatic-associated genes and marker protein, glycogen storage, albumin secretion, and urea production. It is also found that hepatic differentiation of MSCs within the ALS is much more efficient than two-dimensional culture *in vitro*. Importantly, the hepatic-like tissues (HLT) generated by repopulating ALS with MSCs are able to act as functional grafts and rescue lethal hepatic failure after transplantation *in vivo*. In summary, the cryo-chemical method used in this study is suitable for decellularization of liver and create acellular scaffolds that can support hepatic differentiation of MSCs and be used to fabricate functional tissue-engineered liver constructs.

1. Introduction

Liver diseases are one of the leading causes of death worldwide and account for approximately 1–2 million deaths per *annum* according to the World Health Organization. The only curative mode of management for end-stage chronic hepatic diseases is liver transplantation. However, the limited availability of donor organs for transplantation is a major issue in this context [1]. Alternative approaches, such as a stem cell-based regenerative medicine, offer possibilities that help to overcome problems related to the shortage of livers for transplantation [2]. In our previous study, we were able to successfully induce human mesenchymal stem cells (MSCs) to differentiate into hepatocyte-like cells using a two-step protocol *in vitro* [3]. MSCs possess significant potential for hepatic tissue engineering because autologous MSCs can easily be isolated and extensively scaled up [4,5]. In addition, the technique of differentiation of MSCs into hepatocyte-like cells using a two-dimensional (2D) culture system has been well established. However, it has been challenging to find a suitable environment that will allow these multipotent cells to develop toward a more specific state and allow the formation of an implant construct that is able to function well *in vivo*.

Tissue engineering is developing into an advanced area of research that aims at generating biological substitutes which can be used to repair, regenerate or even replace malfunctioning tissues [6]. Scaffolds play a crucial role in providing an appropriate biological environment that includes structural support for cell attachment and subsequent tissue development [7]. Therefore, the scaffold materials must have the chemical and physical properties that support the clinical usefulness of the final tissue. However, the

* Corresponding author. Department of Medical Research and Education, Taipei Veterans General Hospital, Institute of Clinical Medicine, National Yang-Ming University, 201, Sec. 2, Shi-Pai Road, Taipei 11217, Taiwan. Tel.: +886 2 2875 7391; fax: +886 2 2875 7824.
E-mail addresses: windszzz@gmail.com (W.-C. Jiang), ray800201@yahoo.com.tw (Y.-H. Cheng), emh1989@gmail.com (M.-H. Yen), yichang@ym.edu.tw (Y. Chang), vyang@sbumed.org (V.W. Yang), kslee@vghtpe.gov.tw (O.K. Lee).

0142-9612/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2014.01.024

selections of an ideal scaffold for hepatic tissue engineering remains an open question. Previous studies have utilized both synthetic (e.g. polylactic acid, polyglycolic acid, and polyethylene glycol hydrogels) [8–10] and natural (e.g. collagen, alginate and chitosan) [11–14] three-dimensional (3D) scaffolds that provide an environment supporting the maintenance and growth of hepatocytes. More recently, studies have shown that organ decellularization is an attractive strategy because it allows the creation of a naturally occurring 3D biomimetic scaffold that is available for tissue engineering [15,16]. Using this approach, organs such as heart [17], lung [18] and kidney [19] etc, are able to retain most of their native extracellular matrix proteins, bio-molecules and spatial organization. In addition, this 3D architecture provides an optimum vascular structure for oxygen and nutrient diffusion.

In this study, we aim to fabricate acellular liver scaffolds (ALS) from the whole liver using a cryo-chemical decellularized procedure, which is capable of preserving most of the major components of the native liver extracellular matrix and retaining the intact vascular framework [20,21]. Subsequently, by employing a previously developed hepatic differentiation platform [3], the ALS is used as a scaffold for MSCs to differentiate into hepatic-like cells; the functions of MSC-differentiated hepatic-like cells are compared with the traditional 2D cell culture system. Moreover, *in vivo* functionality of MSC-laden ALS is to be tested in a carbon tetrachloride (CCl_4) induced fulminant hepatic failure mice models.

2. Materials and methods

2.1. Fabrication of ALS

The whole livers were harvested from Balb/c mice, which were purchased from the National Laboratory Animal Center (Taipei, Taiwan). After anesthesia with 2.5% Avertin (Sigma–Aldrich, 10 ml/kg body weight), a longitudinal abdominal incision was made in order to expose the liver. The portal vein was cannulated with Polyethylene Tubing-50 and attached to a peristaltic pump (EYELA, Japan). Deionized water was perfused through the portal vein at a rate of approximately 1 ml/min for 1 h and then the liver was frozen at −80 °C for 24 h. Next, the frozen livers were thawed at room temperature and perfused with deionizer water at 1 ml/min for 1 h. Subsequently, 1% Triton-X 100 (Sigma–Aldrich) plus 0.1% ammonium hydroxide (Sigma–Aldrich) in deionizer water was perfused throughout the livers to bring about decellularization and at the rate of 1 ml/min until the perfusate became clarity. Finally, prior to sterilization by gamma irradiation, the acellular liver scaffold that had been created by the above procedures was rinsed with sterile water to remove the remaining decellularization detergent.

2.2. Repopulation of ALS by MSCs

MSCs used in this study were isolated from the bone marrow of Balb/c mice. For repopulation of ALS, 10th- to 12th-passage cells, at 1.0–1.2 × 10^4 cells/cm^2, were maintained in low-glucose Dulbecco's Modified Eagle Medium (Sigma–Aldrich) supplemented with 10% fetal bovine serum and 100 units of penicillin, 1000 units of streptomycin, and 2 mmol/L L-glutamine (Gibco BRL). MSCs were introduced into ALS via portal vein which was retained in ALS and cannulated with Polyethylene Tubing-50 *ex vivo*. Each ALS had culture medium circulated through it via the portal vein by the peristaltic pump (EYELA, Japan) at 1 ml/min for 1 h prior to recellularization. To allow total recellularization using 50 million cells, MSCs were infused into ALS using five steps at 10 min intervals; each step consisting of 10 million cells. After 40 min, the perfusate was collected, and cell viability and retention in the scaffold were determined. The flow rate of medium was used at 1 ml/min for culture of the HLT.

2.3. In vitro hepatic differentiation

To induce hepatic differentiation, MSCs were cultured in ALS and tissue culture dish. Differentiation was induced by treating the MSCs for 4 weeks with a 2-step protocol that we had previously reported [3]. Thereafter, medium replacements were performed twice weekly. Each of study group was following the above procedure for hepatic differentiation.

2.4. Histological analysis

Liver tissues were fixed in 3.7% formaldehyde (Sigma–Aldrich) in phosphate buffered saline (PBS, Gibco BRL) solution overnight. Samples were dehydrated in 30% sucrose in PBS overnight, embedded in O.C.T compound (Sakura), frozen quickly in liquid nitrogen and sectioned at 5 μm thickness. Sample sections were stained using Mayers hematoxylin (Sigma–Aldrich) and counterstained with Eosin-Y (Sigma–Aldrich). Stained sections were observed using an Olympus AX80 microscope (Olympus).

2.5. Scanning electron microscopy

Samples were fixed with 2% glutaraldehyde in PBS for 2 h and post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences) for 1 h and rinsed three times in PBS. The tissue samples were dehydrated through a graded series of ethanol washes, beginning with 50% and progressing through 70%, 90% and 100% absolute ethanol, which were followed by critical point drying using an Emscope CPD 750. Samples were sputter coated with a 7-nm layer of gold-palladium (Cressington 108 sputter coater) and visualized at a voltage of 12 kV using a JEM 6335F field emission gun SEM (JEOL, Peabody, MA).

2.6. DNA content assay

DNA was extracted from a small piece of excised normal livers and ALS using the illustraTM tissue and cells genomic Prep Mini Spin Kit (GE Healthcare) according to the manufacturer's instructions. DNA concentration was determined by spectrophotometer.

2.7. Sirius red/fast green staining for collagen content

Sirius red/fast green solution was prepared using a saturated solution of picric acid (Sigma–Aldrich) in deionizer water (1.2 g per 100 ml) that contained 0.1 g of Direct Red (Sigma–Aldrich) and 0.1 g of Fast Green FCF (Sigma–Aldrich). The solution was allowed to stand for 15 min at room temperature before filtration. After immersing in deionizer water for 5 min, 0.2 ml Sirius Red/Fast Green solution was added to the frozen sections for 30 min. Sections were washed with deionizer water, scraped off the slides, incubated in 2 ml 0.1 N NaOH (Sigma–Aldrich) in methanol for 1 min, and centrifuged. The absorbance of the supernatant was determined at 605 and 540 nm. The value corresponding to 26% of the optical density at 605 nm was calculated, representing the contribution of Fast Green to the absorbance of Sirius Red at 540 nm. The above value was subtracted from the absorbance at 540 nm to obtain the corrected absorbance. Subsequently, the absorbance at 605 nm was divided by 2.08 and the corrected absorbance was divided by 38.4 to obtain the net amount of collagen and non-collagen protein in the section. Total protein was the sum of both values. Results were expressed as the ratio of collagen (μg) to total protein (mg) in order to remove any differences due to variations in the weight of the slices. All analyses were performed in triplicate.

2.8. RNA extraction

Total RNA was isolated using Trizol Reagent (Sigma–Aldrich) as described by the manufacturer's instructions. RNA concentration was determined by spectrophotometer at 260 nm. The purity of the RNA was determined by spectrophotometry at 280 nm and the samples were stored at −80 °C until further use. RNA samples (2 μg) were reverse transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase (MMLV Reverse Transcriptase, Promega).

2.9. Real time RT-polymerase chain reaction

Real time quantitative PCR was performed using an ABI PRISM 7700 (Applied Biosystems) and analyzed using the accompanying software. Target genes that were regulated at the mRNA level during MSCs differentiation were pinpointed and then specific primers targeting these genes were designed using the Primer Express software (Applied Biosystems). DNA-intercalating SYBR green Master Mix reagent (Applied Biosystems) was used to detect the reverse transcribed PCR product. For the reverse-transcription polymerase chain reaction (RT-PCR), the following conditions were used: 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Quantitative real-time RT-PCR (Q-PCR) was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems). The alteration in gene expression was obtained using the △△Ct method in which all samples were first normalized against the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) present in the same sample.

2.10. Periodic acid-schiff (PAS) staining

The differentiated cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 15 min. Samples were then oxidized in 1% periodic acid (Sigma–Aldrich) for 5 min, rinsed three times with deionizer water, treated with Schiff's reagent (Sigma–Aldrich) for 30 min, and then rinsed three times with deionizer water for 5–10 min. Finally, the samples were observed using an Olympus AX80 microscope (Olympus).

2.11. Immunofluorescence staining

The differentiated cells from 2D culture and ALS groups were fixed in 3.7% formaldehyde in PBS and permeabilized in 0.1% Triton X-100 for 15 min at room temperature. After permeabilization, the samples were blocked with 2% bovine serum albumin (BSA, Sigma–Aldrich) in PBS for 1 h and thereafter treated with primary antibodies overnight at 4 °C diluted in blocking buffer. The antibodies used in this study were mouse polyclonal anti-collagen type I (COL-I, 1:250 Abcam),

Please cite this article in press as: Jiang W-C, et al., Cryo-chemical decellularization of the whole liver for mesenchymal stem cells-based functional hepatic tissue engineering, Biomaterials (2014), http://dx.doi.org/10.1016/j.biomaterials.2014.01.024