Journal of Biotechnology 174 (2014) 39–48

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Hepatic differentiation of human embryonic stem cells on microcarriers

Yonsil Park, Yemiao Chen, Laura Ordovas, Catherine M. Verfaillie*

Stem Cell Institute Leuven, Department of Development and Regeneration, Cluster Stem Cell Biology and Embryology, KU Leuven Medical School, Belgium

ARTICLE INFO

Article history:
Received 19 October 2013
Received in revised form
23 December 2013
Accepted 14 January 2014
Available online 27 January 2014

Keywords:
Human embryonic stem cells
Microcarrier
Hepatic differentiation
Bioreactor
Suspension culture system

ABSTRACT

Translation of stem cell research to industrial and clinical settings mostly requires large quantities of cells, especially those involving large organs such as the liver. A scalable reactor system is desirable to ensure a reliable supply of sufficient quantities of differentiated cells. To increase the culture efficiency in bioreactor system, high surface to volume ratio needs to be achieved. We employed a microcarrier culture system for the expansion of undifferentiated human embryonic stem cells (hESCs) as well as for directed differentiation of these cells to hepatocyte-like cells. Cells in single cell suspension were attached to the bead surface in even distribution and were expanded to 1×10^6 cells/ml within 2 days of hESC culture with maintenance of the level of pluripotency markers. Directed differentiation into hepatocyte-like cells on microcarriers, both in static culture and stirred bioreactors, induced similar levels of hepatocyte-like cell differentiation as observed with cells cultured in conventional tissue culture plates. The cells expressed both immature and mature hepatocyte-lineage genes and proteins such as asialoglycoprotein receptor-1 (ASGPR-1) and albumin. Differentiated cells exhibited functional characteristics such as secretion of albumin and urea, and CYP3A4 activity could be detected. Microcarriers thus offer the potential for large-scale expansion and differentiation of hESCs induced hepatocyte-like cells in a more controllable bioreactor environment.

1. Introduction

Among many organs and tissues for which cell therapies are being pursued, the liver is an organ that lacks effective therapies for liver failure. The liver is the largest organ in the adult body and has major functions in metabolism and detoxification, playing a role in protein synthesis, glucose metabolism and lipid metabolism. Existing treatments for liver failure are limited, and the only real cure is liver cell or organ transplantation. The shortage of donor cells and organs often makes it unreachable for a large number of patients. Hepatocytes isolated from liver and cultured *in vitro* have limited proliferation potential and often very quickly lose their functional attributes. Therefore, investigators are looking into the possibility to generate hepatocytes from progenitor cells or stem cells. However, any therapeutic application requires a large quantity of cells in the order of $>10^9$–10^{10} cells per treatment. In addition to the clinical need for hepatic cell transplantation, human hepatocytes attract attention from the pharmaceutical industry for drug toxicity and metabolism testing to better estimate acute and chronic toxicity, which should decrease the failure of drugs in clinical trials. The shortage of human livers also makes it difficult to use primary human hepatocytes for pharmaceutical purposes.

The cultivation of hepatic progenitor cells, immortalization of hepatocytes, and derivation of functional hepatocytes from stem cells are among the more prevalent approaches (Agarwal et al., 2008; Allain et al., 2002; Cai et al., 2000; Hay et al., 2008; Herrera et al., 2006; Wege et al., 2003). Especially, the progress in stem cell science has opened the possibility that many types of stem cells including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), pluripotent adult stem cells, hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) can be used to produce hepatocyte-like cells (Zhang et al., 2012). Hepatic cells derived from pluripotent stem cells (PSCs), which are capable of unlimited self-renewal have great potential to be such a steady cell source.

For stem cells to be widely applied to clinical and pharmaceutical fields, having a desired cell type as well as a sufficient supply of those cells should be fulfilled. The conventional method of expanding and differentiating PSCs in tissue culture plates is labor intensive and difficult to scale up. Employing a scalable reactor system can enable ample supply of differentiation competent or differentiated cells to meet such a clinical need. Various reactor systems have been investigated to support stem cell expansion and differentiation toward hepatocytes; rotary culture systems,

* Corresponding author at: UZ Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium. Tel.: +32 16 330295.
E-mail address: catherine.verfaillie@med.kuleuven.be (C.M. Verfaillie).

0168-1656/$ – see front matter © 2014 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jbiotec.2014.01.025

perfusion culture systems, and stirred suspension systems have been employed for hepatic cell cultures (Matsumoto et al., 2008; Miki et al., 2011; Wang et al., 2012; Yin et al., 2007). Among those systems, stirred suspension culture has the advantage of having been widely used in growing mammalian cells for decades. It also allows high-density culture in a more homogeneous environment, and should allow one to monitor and control culture parameters. Cells are suspended in culture either as spheroids or with the support of scaffold. Stirred reactor cultures have been used to expand or differentiate hematopoietic stem and progenitor cells (HSPCs), neural stem cells (NSCs), and human and mouse embryonic stem cell (ESCs) grown as embryoid bodies (EBs) (Cameron et al., 2006; Fok and Zandstra, 2005; Gilbertson et al., 2006; Li et al., 2006).

Microcarriers have also been used as scaffold to support differentiation in stirred suspension culture. Microcarriers are particles, typically made of cellulose, glass, plastic, or polyester, and usually with diameters of 100–250 μm. Microcarriers have the advantage of providing a large surface area for a given reactor volume. By providing surface for attachment, they allow anchorage dependent cells to be cultivated in stirred bioreactors for a large-scale. Undifferentiated PSCs were seeded onto the beads, expanded further, and differentiated into different cell types, including neural progenitor cells and cardiomyocytes (Bardy et al., 2013; Lecina et al., 2010; Rodrigues et al., 2011). Few studies have assessed hepatic differentiation on microcarriers. Rat multipotent adult progenitor cells (rMAPCs) have been expanded and differentiated on dextran beads in a spinner system to yield hepatocyte-like cells expressing early and mature hepatic genes (Park et al., 2010). hESCs have also been expanded on different types of beads and differentiated into endodermal cells (Lock and Tzanakakis, 2009), showing the potential of scaled-up culture of ESC hepatic differentiation using microcarrier-based suspension culture. Although most micro-porous microcarrier cultures are still 2D, the stirred bioreactor allows more dynamic culture environment than in conventional 2D culture in tissue culture plates. In this study, using an established hepatic differentiation protocol (Roelandt et al., 2012, 2010), we show that hESCs can be differentiated into hepatocyte-like cells on dextran microcarriers in suspension culture system. Microcarrier culture has the potential of being an efficient scalable means of undifferentiated hESC expansion for a short period and directed differentiation toward hepatocytes.

2. Materials and methods

2.1. hESC culture

The human H9 cell line was used in this study, with approval of the ethics committee at KU Leuven. H9 cells were cultured in a feeder free condition on hESC qualified Matrigel™ (BD) coated surface and with mTeSR1™ (Stem cell technologies) medium.

2.2. hESC cell seeding onto microcarriers for suspension culture

Cytodex, a crosslinked dextran microbead, is microporous, with a void fraction greatly exceeding 90%, and supports cell growth on the external surface of the bead. Cytodex 1 and Cytodex 3 microcarriers were purchased from GE Healthcare as dry powder. Collagen based microcarriers, Cultispher and Spherecol, were tested for cell seeding and differentiation. Cultispher S (Sigma) are macroporous gelatin microcarriers. Spherecol® (Advanced BioMatrix) are purified collagen beads. The microcarrier stock was prepared by washing 1 g of microcarrier powder with PBS for 3–5 times. Swollen beads were sedimented in 100 ml fresh PBS and sterilized by autoclaving.

Prior to seeding the hESC, microcarriers were washed with DMEM/F12 (Life technologies) twice. Matrigel with reduced growth factors (BD) was diluted in DMEM/F12 in 1:5 and added to the washed beads to make a final dilution 1:10. Beads were stirred in the Matrigel solution for 2 h at 37 °C. hESC cultures in 6 well plates (Corning) were washed with PBS. 1 ml Accutase (Sigma) was added to each well, and the plates were incubated at 37 °C for 5 min. The plates were lightly tapped several times and gently pipetted to make single cell suspension. 2 ml of DMEM/F12 was added to each well to neutralize accutase. The cells were collected, centrifuged, and resuspended in mTeSR1 medium at a concentration of 5×10^6 cells/ml. Cells were added to 25 mg/ml Cytodex microcarriers at the concentration of 5×10^6 cells/ml in 5 ml culture medium. The mixture was incubated for 2 h at 37 °C to allow initial cell attachment to the beads with occasional shaking of the tube. Then the mixture was transferred into either a low attachment plates (Corning) or a spinner flask (Bellco). The culture was stirred at 20–25 rpm on a magnetic stir plate (2mag) and maintained at 37 °C, 21% O_2 tension and 5% CO_2 for 2–3 days of expansion with medium change every day. 90% of medium was removed carefully when cell-laden beads were sedimented, and then 100% of fresh medium was added to the flask.

2.3. In vitro hepatic differentiation

hESCs were differentiated into hepatocyte-like cells using a four step hepatic differentiation protocol developed in Verfaillie lab (Roelandt et al., 2012). For static plate differentiation, cell laden beads were inoculated in an ultra-low attachment 6-well plate (Corning) at a starting cell concentration of 1.0×10^6 cells/ml. After 2 days of expansion culture, the cells reached confluency on the microcarrier surface, they were washed once with PBS and resuspended in hepatic differentiation medium (2 ml of differentiation medium per well for static differentiation and 50 ml in a 250 ml spinner flask for stirred suspension differentiation). The composition of serum free differentiation basal medium is a 60/40 (v/v) mixture of low glucose Dulbecco's Modified Eagle media (DMEM) (Life technologies) and MCDB-201 (Sigma) supplemented with 0.026 μg/ml ascorbic acid 3-phosphate (Sigma), linoleic acid bovine serum albumin (LA-BSA, Sigma) (final concentrations of 0.25×10^3 μg/ml BSA and 2.03 μg/ml linoleic acid), insulin-transferrin-selenium (ITS, Sigma) (final concentration 2.5 μg/ml insulin, 1.38 μg/ml transferring, 0.0012 μg/ml sodium selenite), 0.4 μg/ml dexamethasone (Sigma), 4.3 μg/ml β-mercaptoethanol (Sigma). The growth factor supplements were added with the 90% medium change as follows: (i) day 0: Activin A (100 ng/ml) and Wnt3a (50 ng/ml); (ii) day 2: Activin A (100 ng/ml); (iii) day 4: BMP4 (50 ng/ml); (iv) day 8: aFGF (50 ng/ml); (v) day 12: HGF (20 ng/ml). Differentiations were carried out at 21% O_2 and 5% CO_2, 37 °C for 18 days with 60% media change every 2 days corresponding to the differentiation stage.

2.4. RNA isolation and quantitative real time polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cell lysates using the RNAeasy minikit (Sigma) according to instructions provided in the kit. cDNA was synthesized from the extracted RNA using the Super-script III reverse transcriptase (Life technologies) method. The PCR mix consisted of cDNA samples, SYBR Green Mix PCR reaction buffer (Life technologies) and primers (5 μM stocks, sequences in Table 1). The RT-qPCR reaction was run on a Realplex mastercycler (Eppendorf) using the following program: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min followed by a dissociation protocol to obtain a melting curve. Transcription abundance relative to *GAPDH* was calculated as ΔCt which is