Biochemical Engineering Journal 60 (2012) 67–73

Contents lists available at SciVerse ScienceDirect

Biochemical Engineering Journal

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

Enhanced liver functions in mouse hepatoma cells by induced overexpression of liver-enriched transcription factors

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ARTICLE INFO

Article history:
Received 21 July 2011
Received in revised form 7 October 2011
Accepted 13 October 2011
Available online 20 October 2011

Keywords:
Animal cell culture
Biomedical
Recombinant DNA
Hepatoma cell
Liver-enriched transcription factor
Liver function

ABSTRACT

Hepatoma cells, which are derived from liver carcinoma, are able to proliferate infinitely under culture conditions. However, the liver functions of hepatoma cells are generally low compared with those of hepatocytes in a liver. Here, we attempted to create genetically engineered hepatoma cells with enhanced liver functions by overexpression of liver-enriched transcription factors (LETFs), which are associated with the transcription of liver-specific genes and hepatic differentiation. For this purpose, genes for eight LETFs, hepatocyte nuclear factor (HNF)-1α, HNF-1β, HNF-3β, HNF-4α, HNF-6, CCAAT/enhancer binding protein (C/EBP)-α, C/EBP-β and C/EBP-γ, were obtained from the mouse liver. Mouse hepatoma Hepa1-6 cells were transduced with retroviral vectors, in which inducible expression cassettes for the LETF genes were introduced. Cell clones with inducible expression of high liver functions were established. Upon overexpression of the LETF genes, cell proliferation ceased and the cells exhibited an epithelial morphology, indicating hepatic maturation of hepatoma cells. This approach for genetic modification of hepatoma cells may be promising for the construction of cells for use in bioartificial liver support systems.

1. Introduction

Liver failure is one of the major causes of death around the world [1], because the liver is multifunctional and a vital organ for survival. Although liver transplantation is the only effective treatment for acute liver failure [2], it has several limitations including the shortage of organ donors. In recent years, bioartificial liver (BAL) support systems, in which functional hepatic cells such as primary hepatocytes are placed in a bioreactor to cover the hepatic functions through blood circulation between the bioreactor and a liver failure patient [3], have been expected to make up for the organ donor shortage.

Although normal human hepatocytes are the ideal cell source for BAL systems, it is difficult to culture primary hepatocytes for long periods of time with the current cell culture technology and to obtain sufficiently large numbers of differentiated human hepatocytes for practical application of BAL systems. Therefore, embryonic stem cells, hepatic stem cells and bone marrow cells, which possess proliferation ability and retain their differentiation potential, have been considered as alternative cell sources for large-scale hepatocyte preparation [4]. However, the efficiency and degree of differentiation in these cells under culture conditions are not presently high and are insufficient to obtain large numbers of functional hepatocytes. Moreover, cultures for the proliferation and differentiation of such stem cells using cytokines are often time-consuming and costly processes.

Hepatoma cells derived from liver carcinoma are one of the alternative cell sources for BAL systems. Hepatoma cells can be cultured easily and inexpensively compared with the stem cells described above. Hepatoma cells also express some liver functions under ordinary culture conditions, because they are derived from hepatocytes. However, the expression levels of the liver functions in hepatoma cells are generally very low compared with those of hepatocytes in a liver.

Liver-enriched transcription factors (LETFs) play critical roles in liver development and maintenance of the hepatocyte phenotype through transcriptional regulation of liver-specific gene expressions. Several LETFs are always expressed in hepatocytes and their combined actions appropriately control the gene expressions that are necessary to maintain various hepatic functions, such as albumin secretion, urea synthesis and drug metabolisms, at the transcriptional level [5]. In the present study, we attempted to create genetically engineered hepatoma cells with enhanced liver functions by overexpression of LETFs. For this purpose, we selected eight transcription factors, hepatocyte nuclear factor (HNF)-1α, HNF-1β, HNF-3β, HNF-4α, HNF-6, CCAAT/enhancer binding protein (C/EBP)-α, C/EBP-β and C/EBP-γ, based on their known functions in hepatocytes [6,7], and inducible expression cassettes for these LETF genes were introduced into hepatoma cells by retroviral transduction.

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doi:10.1016/j.bej.2011.10.004

2. Materials and methods

2.1. Cell culture

Mouse hepatocellular carcinoma Hepa1-6 cells (Riken BioResource Center, Tsukuba, Japan) were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 0.1 mg/mL streptomycin sulfate and 100 U/mL penicillin G potassium (Wako Pure Chemical Industries, Osaka, Japan). 293FT cells (Invitrogen, Carlsbad, CA, USA) were used as producer cells for retroviral vectors based on mouse stem cell virus (MSCV) [8], and were cultured in high-glucose DMEM supplemented with 10% FBS, 0.1 mM MEM non-essential amino acids (Invitrogen) and 20 mM HEPES (Dojindo, Kumamoto, Japan) in collagen-coated tissue culture dishes (Asahi Techno Glass, Tokyo, Japan). Parenchymal hepatocytes were obtained from female C57BL/6NCrSlc mice (8 weeks old) by the collagenase perfusion method. The animal experiment was approved by the Ethics Committee for Animal Experiments of the Faculty of Engineering, Kyushu University (A23-146-0). Hepatocytes were seeded into the wells of 24-well collagen-coated plates (Asahi Techno Glass) at a density of 1.0×10^5 cells/well and cultured in Williums' medium E (Invitrogen) supplemented with 5% FBS, 0.1 μM $CuSO_4 \cdot 5H_2O$, 25 nM Na_2SeO_3, 1.0 μM dexamethasone, 0.1 μM insulin, 20 ng/mL EGF (Biomedical Technologies, Stoughton, MA, USA), 0.1 mg/mL streptomycin sulfate and 100 U/mL penicillin G potassium. Liver functions of hepatocytes were measured on days 1–2 of culture. All cells were cultured at 37 °C in a 5% (v/v) CO_2 incubator.

2.2. Cloning of mouse LETFs and construction of retroviral vector plasmids

Total RNA was extracted from the mouse liver using a commercially available kit (RNAiso plus; Takara, Otsu, Japan) according to the manufacturer's protocol. The isolated RNA was reverse-transcribed with an oligo-dT primer using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) to produce cDNA. The coding regions of the mouse LETF genes were amplified by PCR from the cDNA using KOD-plus DNA polymerase (Toyobo) and the primer pairs listed in Supplementary Table 1. The primers included restriction site sequences (forward primers, *Xho*I or *Sal*I site; reverse primers, *Bam*HI site) to append them to the ends of each PCR product to facilitate subsequent subcloning. The PCR amplifications were performed at 94 °C for 2 min, followed by 35 cycles of amplification at 94 °C for 15 s, various annealing temperatures (Supplementary Table 1) for 30 s and 68 °C for 60–90 s. For HNF-1α, the amplification of the sequence was divided into two parts because of the length of the sequence. The PCR products of LETFs except for HNF-1α and HNF-4α were digested with *Xho*I and *Bam*HI and ligated into *Xho*I- and *Bam*HI-digested pBluescriptII KS (−) (Toyobo). The PCR products of HNF-1α and HNF-4α were digested with *Sal*I and *Bam*HI and ligated into *Sal*I- and *Bam*HI-digested pBluescriptII KS (−). The DNA sequences of the PCR products were confirmed using a DNA sequencer (Prism 3130 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) and compared with their sequences in the GenBank database.

MSCV-based retroviral vectors were used for gene transfer into Hepa1-6 cells. The Tet-On system (Clontech, Mountain View, CA, USA) was incorporated into the retroviral vectors for inducible expression of the LETF genes. For retroviral vector production, retroviral vector plasmids encoding expression cassettes for the reverse tetracycline-dependent transactivator (rtTA) and LETF genes were constructed. The pQMSCV/EGFP-CMV-rtTA-WPRE plasmid encoded a constitutive expression cassette for rtTA that was activated by the addition of doxycycline (Dox). The pQMSCV/EGFP-TREtight-LETF-WPRE plasmids encoded an expression cassette for each LETF including a tetracycline-responsive element, such that LETF expression was promoted by the activated rtTA. These plasmids included an enhanced green fluorescent protein (EGFP) gene under the control of viral LTR promoters for evaluation of the viral titer. To construct pQMSCV/EGFP-CMV-rtTA-WPRE, a DNA fragment of the EGFP gene was amplified from pIRES-EGFP (Clontech) by PCR using the primers 5′-ACCGAATTCACACGATGATAATATGGCCAC-3′ (forward, *Eco*RI site (underlined)) and 5′-ATTCTCGAGAGGCCGCTTTACTTGTACAG-3′ (reverse, *Xho*I site (underlined)). The PCR fragment was ligated into *Eco*RI- and *Xho*I-digested pQMSCV/CMV-VEGF-IRES-EGFP [9] to generate pQMSCV/EGFP. Next, the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequence from pMSCV/GΔAhEpoW [10] was inserted into ClaI-digested pQMSCV/EGFP, resulting in pQMSCV/EGFP-WPRE. Thereafter, pQMSCV/EGFP-CMV-rtTA-WPRE was generated by ligation of the cytomegalovirus (CMV) promoter and the rtTA sequence from pTet-On Advanced (Clontech) into *Xho*I- and *Cla*I-digested pQMSCV/EGFP-WPRE. For the construction of pQMSCV/EGFP-TREtight-LETF-WPRE, the tetracycline-responsive promoter from pTRE-Tight (Clontech) was ligated into *Xho*I- and *Cla*I-digested pQMSCV/EGFP-WPRE to generate pQMSCV/EGFP-TREtight-WPRE. The pQMSCV/EGFP-TREtight-LETF-WPRE plasmids were then generated by ligation of the *Xho*I (or *Sal*I)- and *Bam*HI-digested DNA fragments encoding each LETF gene into *Xho*I- and *Bam*HI-digested pQMSCV/EGFP-TREtight-WPRE. pQMSCV/EGFP-TREtight-DsRed-WPRE was constructed by insertion of a DNA fragment encoding DsRed protein from pIRES2-DsRed-Express2 (Clontech) into *Xho*I- and *Bam*HI-digested pQMSCV/EGFP-TREtight-WPRE.

2.3. Retroviral vector production and infection

Retroviral vectors encoding the rtTA and LETF genes were produced as described previously [11]. Culture medium containing retroviral particles was filtered to remove cell debris using a 0.45-μm cellulose acetate filter (Advantec, Tokyo, Japan). The viral solution was then concentrated by centrifugation ($60{,}000 \times g$ at 4 °C for 2 h). After careful removal of the supernatant, the viral pellet was suspended in 50 mM Tris–HCl buffer (pH 7.8) containing 130 mM NaCl and 1 mM EDTA. For retroviral infection, cells were plated in the wells of 24-well tissue culture plates (Greiner Bio-One, Frickenhausen, Germany) at a density of 1.5×10^4 cells/well. After 1 day of culture, the medium was replaced with fresh medium containing concentrated viral solutions and 8 μg/mL polybrene (Sigma–Aldrich). The cells were incubated at 37 °C for 6 h, and the medium was then changed to fresh medium. The transduction titer was determined using Hepa1-6 cells by a serial dilution method after 48 h of infection [11].

2.4. Albumin secretion assay

Hepatoma cells were plated in the wells of 24-well tissue culture plates at a density of 4.0×10^4 cells/well. From the next day (day 0), the cells were cultured in the presence or absence of 0.1 μg/mL Dox (Sigma–Aldrich). The medium was changed every other day. On day 7, the culture medium was collected and stored at −80 °C for subsequent analysis. The secreted albumin for 24 h was measured by ELISA using a commercially available kit (Mouse Albumin ELISA Quantitation Set; Bethyl, Montgomery, TX, USA) according to the manufacturer's protocol.

2.5. Ammonia and urea metabolism analysis

Hepatoma cells were seeded into the wells of 24-well tissue culture plates at a density of 4.0×10^4 cells/well in medium with