Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Primary isolated hepatic oval cells maintain progenitor cell phenotypes after two-year prolonged cultivation

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Background & Aims: Although expandable hepatic progenitors provide renewable cell sources for treatment of hepatic disorders, long-term cultivation of hepatic progenitors may affect proliferation and differentiation abilities, and even initiate the formation of malignant cancer stem cells. This study aims to determine characteristics of primary cultured hepatic oval cells after prolonged cultivation *in vitro*.

Methods: Hepatic oval cells isolated from rats fed with a choline-deficient, ethionine-supplemented diet were continuously propagated every 5–7 days, to 100 passages over two years. Hepatocytic differentiation was induced by sodium butyrate and characterized using western blot, periodic acid Schiff assays, albumin secretion and urea production. Proliferation capacity was evaluated using growth-curve and cell-cycle analysis; anchorage-independent growth and tumorigenicity were determined using soft agar and xenograft assay.

Results: After 2 years of serial passages, hepatic oval cells with typical epithelial morphology continuously expressed OV-6, BD-1, BD-2, and Dlk as markers for hepatic progenitors, cytokeratin 19 as a cholangiocyte marker, and alpha-fetoprotein and albumin as hepatocyte markers. Furthermore, sodium butyrate could induce these cells to become glycogen-storage cells with the functions of albumin secretion and ureagenesis from ammonia clearance, indicating hepatocytic differentiation. Although proliferation slightly accelerated after the 50th passage, hepatic oval cells stayed diploid cells with features of chromosomal stability, which did not acquire anchorage-independent growth capacity and caused no tumor in immunodeficient mice, suggesting no spontaneous malignant transformation.

Conclusions: Hepatic oval cells retain the progenitor cell features without spontaneous malignant transformation after prolonged cultivation, and thus may serve as an expandable cell source for future exploitation of stem cell technology.

Keywords: Hepatic progenitors; Long-term cultivation; Proliferation; Malignant transformation.

Received 12 November 2009; received in revised form 7 May 2010; accepted 7 May 2010; available online 15 July 2010

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Abbreviations: CDE, choline-deficient ethionine-supplemented diet; HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; ALB, albumin; CK19, cytokeratin 19; NaBu, sodium butyrate.

Introduction

Development of cell therapy-based strategies for the treatment of liver failure and of inherited metabolic diseases has become a necessity because of the limitations of orthotropic liver transplantation, including shortage of donor livers [1–4]. This shortage also limits the availability of hepatocytes. To date, few studies have succeeded in long-term culture and repeated cryopreservation of primary hepatocytes, which usually can only survive for several weeks while gradually losing liver-specific functions [5]. A wider use of these techniques will not be possible until adequate numbers of functional cells for transplantation become more readily available [4]. In recent years, numerous articles have reported about the generation of liver cells or "hepatocyte-like cells" from hepatic progenitor/oval cells or cell lines that exhibit stem cell properties [1]. This could provide exciting new opportunities for cell therapy as these stem/progenitor cells proliferate efficiently *in vitro* and may therefore help to generate a larger supply of hepatocytes for transplantation. However, the fact that oval cell activation precedes the development of hepatocellular carcinoma (HCC) in almost all models of hepatocarcinogenesis, and invariably accompanies late-stage cirrhosis in humans (a condition characterized by long-standing hepatocyte damage and chronic inflammation leading to fibrosis [6,7]), raises the possibility that hepatocellular carcinomas (HCC) arise from liver stem/progenitor cells [8–10]. Accumulating evidence suggests that the gene expression profiling in a subset of HCCs is consistent with that of hepatic progenitors [11–16], which make a deadly mix of stem cells and cancer. Hepatic progenitors' apparent ability to develop liver tumors is a potentially important drawback for the treatment of liver diseases with stem or progenitor cells. Obtaining enough cells for cell therapy requires continuous propagation of hepatic stem/progenitor cells based on their proliferation capacity *in vitro*; therefore, a precise characterization of stem/progenitor cell populations after serial passages is of utmost importance for the future exploitation of stem cell technology. Despite its significance, few studies focus in detail on whether long-term cultivation of hepatic stem/progenitor cells affects their progenitor cell characteristics, including

immunophenotypes, proliferation ability and differentiation potential or even initiates spontaneously malignant cancer stem cells. Therefore, the aim of this study was to determine whether hepatic oval cells maintained the characteristics of hepatic progenitors and survived without tumorigenicity after serial propagation.

In the present study, primary cultured rat hepatic oval cells were continuously propagated to 100 passages for 2 years. These cells maintained the gene expression profiles of hepatic progenitors without the loss of their hepatocytic differentiation potential. Furthermore, serial passages did not cause spontaneous malignant transformation of these cells, which supports the notion that neither serial passages nor continuous proliferation is the direct causation of malignant transformation of hepatic progenitors. Therefore, hepatic oval cells without immortalized manipulation may serve as an expandable cell source for future basic researches and cell-therapy utilizations.

Materials and methods

Animals and diets

Male Sprague–Dawley rats (130–150 g) were purchased from Beijing Experimental Animal Center (Beijing, China) and allowed access to a choline-deficient diet supplemented with 0.1% wt/wt DL-ethionine (CDE, Sigma–Aldrich, Saint Louis, MO, USA) for 6 weeks. All procedures were performed in accordance with the guidelines of the Chinese Council on Animal Care and with the approval of the Ethics Committee of Capital Medical University (Beijing, China) [17].

Isolation and culture of hepatic oval cells

Hepatic oval cells were prepared and cultured in DMEM/Ham's F12 (Gibco, Grand Island, NY, USA) supplemented with 10% FBS, 0.5 U/ml insulin, 1 ng/ml epidermal growth factor (EGF, PeProTech, Rehovot, Israel), 0.5 ng/ml stem cell factor (SCF, PeProTech), 100 U/ml penicillin and streptomycin as described by Wang et al. [17]. For hepatocyte maturation, the hepatic oval cells were kept in DMEM/F12 medium mentioned above, containing 0.75 mmol/L sodium butyrate (NaBu, Sigma–Aldrich) as described previously [18].

Indirect immunofluorescence cytochemistry

For flow cytometry analysis, cells were detached and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. After being permeabilized with 0.1% saponin in PBS and blocked by normal mouse serum, cells were incubated with one of the primary antibodies (mouse anti-rat OV-6 antibody, mouse anti-rat bile duct antibody-1 [BD-1], or mouse anti-rat BD-2 antibody) at 37 °C for 60 min. After three washes with 0.1% saponin in PBS, the primary antibodies were detected with the corresponding fluorescein isothiocyanate (FITC)-conjugated anti-IgG (BD PharMingen, San Diego, CA, USA). After three washes with 0.1% saponin in PBS, cell fluorescence was analyzed with a FACSCalibur flow cytometer (FACS, Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest software (BD Bioscience). For surface antigen Dlk detection, the cells without permeabilization were blocked by normal rabbit serum, incubated with rabbit anti-Dlk antibody (Abcam, Hong Kong) and FITC-conjugated anti-rabbit IgG (Abcam). In the FACS results, the red line represented the positive signals, while the blue line represented the isotype as background control.

For immunofluorescence microscopy, hepatic oval cells, HepG2 cells and primary cultured hepatocytes were prepared by a two-step collagenase perfusion and repeated centrifugation at 50*g* on glass slides and were fixed with 4% paraformaldehyde in PBS at room temperature for 30 min. After being permeabilized with 0.3% Triton X-100 in PBS and blocked by normal serum, the cells were incubated with rabbit anti-albumin (ALB, diluted 1:200, Bethyl, Montgomery, TX USA), or mouse anti-alpha-fetoprotein (AFP, diluted 1:200, R&D Systems, Minneapolis, MN USA) at 4 °C overnight. After three washes in PBS, the primary antibodies were detected with the corresponding FITC- or Alex Fluor 594-conjugated anti-IgG (Molecular Probes, Eugene, OR, USA) at 37 °C for 20 min. Sections were examined under a Nikon 50i fluorescence microscope (Nikon, Japan). All cell counts were performed on blind-coded samples. Cell count was performed on three independent experiments based on two characteristics: DAPI-positive nuclei and immunoreaction to different markers within the same field. Data are expressed as mean ± SD.

Real-time RT-PCR

Total RNA was extracted from 1×10^6 cells with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The mRNA was reverse-transcribed to the cDNA by a reverse transcript kit (Promega, San Luis Obispo, CA, USA). Quantitative RT-PCR was performed on an ABIPrism 7300 Sequence Detector (Applied Biosystems, Foster City, CA, USA) using the ABI Power SYBR Green PCR Master Mix kit (ABI Applied Biosystem, Warrington, UK). The primers used for amplification (listed in Table 1) were designed to be between 60 and 150 bp in length according to PE Applied Biosystems guidelines for comparative C_t method. The optimum final primer concentration to be used in all conditions was assessed to be 300 nM, to ensure no non-specific amplification occurred in the sample wells. Reverse transcription was carried out for 2 min at 50 °C before 10 min at 95 °C to inactivate the reverse transcriptase which otherwise interferes with the DNA polymerase. Forty cycles of 95 °C for 15 s followed by 60 °C for 60 s were carried out and fluorescence was collected at the end of each extension cycle. The amount of target, normalized to an endogenous reference (GAPDH) and relative to a calibrator (primary cultured oval cells), was given by $2^{-\Delta\Delta C_t}$ as determined by the ABIPrism 7300 System Software's built-in algorithm using an adaptive baseline to determine the C_t. The relative amounts were expressed as mean ± SD from three independent experiments.

Western blot

Expression of AFP, ALB, cytokeratin 19 (CK19) and β-actin was detected by standard western blot procedure described previously [17]. Briefly, total protein was extracted and determined using a Micro BCA™ protein assay reagent kit (Pierce,

Table 1. Primers used in this study.

Gene	Primer sequence	Product size	Accession #
AFP	Sense: 5′-GGAGAAGTGCTGCAAAGACC-3′	120 bp	NM_012493
	Antisense: 5′-TTGTCCTTTCTTCCTCCTGG-3′		
ALB	Sense: 5′-AGAACCAGGCCACTATCTC-3′	110 bp	NM_134326
	Antisense: 5′-CAGATCGGCAGGAATGTTGT-3′		
CK19	Sense: 5′-CAGCAGTATTGAAGTCCAGC-3′	139 bp	NM_199498
	Antisense: 5′-TCAAGCAGGCTTCGGTAGGT-3′		
GAPDH	Sense: 5′-CCTGCCAAGTATGATGACATCAAGA-3′	75 bp	BC059110
	Antisense: 5′-GTAGCCCAGGATGCCCTTTAGT-3′		