The International Journal of Biochemistry & Cell Biology 43 (2011) 1483–1489

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

The HepaRG cell line is suitable for bioartificial liver application

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ARTICLE INFO

Article history:
Received 20 January 2011
Received in revised form 14 June 2011
Accepted 18 June 2011
Available online 24 June 2011

Keywords:
Bioartificial liver
HepaRG
DMSO
Ureagenesis
Metabolism
Carbamoyl-glutamate

ABSTRACT

For bioartificial liver application, cells should meet the following minimal requirements: ammonia elimination, drug metabolism and blood protein synthesis. Here we explore the suitability of HepaRG cells, a human cell line reported to differentiate into hepatocyte clusters and surrounding biliary epithelial-like cells at high density and after exposure to dimethyl sulfoxide (DMSO). The effect of carbamoyl-glutamate (CG), an activator of urea cycle enzyme carbamoylphosphate synthetase (CPS) was studied additionally.

The effects of DMSO and/or CG were assessed in presence of $^{15}NH_4Cl$ on HepaRG cells in monolayer. We tested hepatocyte-specific functions at transcript and biochemical level, cell damage parameters and performed immunostainings.

Ureagenesis, ammonia/galactose elimination and *albumin*, *glutamine synthetase* and *CPS* transcript levels were higher in −DMSO than +DMSO cultures, probably due to a higher cell content and/or cluster-neighbouring regions contributing to their functionality. DMSO treatment increased *cytochrome P450* (*CYP*) transcript levels and CYP3A4 activity, but also cell damage and repressed hepatic functionality in cluster-neighbouring regions. The levels of ammonia elimination, apolipoprotein A-1 production, and transcription of *CYP3A4*, *CYP2B6* and *albumin* reached those of primary hepatocytes in either the + or −DMSO cultures. Preconditioning with CG increased conversion of $^{15}NH_4Cl$ into ^{15}N-urea 4-fold only in −DMSO cultures.

Hence, HepaRG cells show high metabolic and synthetic functionality in the absence of DMSO, however, their drug metabolism is only high in the presence of DMSO. An unparalleled broad hepatic functionality, suitable for bioartificial liver application, can be accomplished by combining CG treated −DMSO cultures with +DMSO cultures.

1. Introduction

Bioartificial livers (BALs) have been developed to temporarily support patients with severe liver failure (Carpentier et al., 2009). BALs are based on a bioreactor with functional liver cells, ideally human proliferative cells with hepatic function. This human bio-component should detoxify accumulating toxic compounds via the cytochrome P450 (CYP) system, maintain metabolic homeostasis and synthesize blood proteins. An important function is the elimination of ammonia, a neurotoxin accumulating during severe liver failure (Butterworth et al., 1987). The liver removes ammonia primarily through the urea cycle and secondarily through fixation into amino acids, particularly glutamine by glutamine synthetase (GS).

Frequently HepG2 cells have been applied as human proliferative bio-component in BALs (Chamuleau et al., 2005). Although HepG2 and subclone C3A show hepatic functionality, like synthesis of blood proteins, the urea cycle is not functional, resulting in absence of ammonia detoxification via this route (Mavri-Damelin

Abbreviations: DMSO, dimethylsulfoxide; CG, carbamoyl-glutamate; CPS, carbamoylphosphate synthetase; CYP, cytochrome P450; BAL, bioartificial liver; GS, glutamine synthetase; ARG, arginase; PHHs, primary human hepatocytes; CAR, constitutive androstane receptor; ALB, albumin; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; DFB, 3-[(3,4-difluorobenzyl)oxy]-5,5-dimethyl-4-[4-(methylsulfonyl) phenyl]furan-2(5*H*)-one; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; APOA1, apolipoprotein A1; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; DFH, 3-hydroxy-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]furan-2(5*H*)-one; DAPI, 4,6-diaminidino-2-phenylindole; HNF4A, hepatocyte nuclear factor 4 alpha; PXR, pregnane X receptor; TF, transferrin; AFP, alpha-fetoprotein; ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; OTC, ornithine transcarbamoylase; ns, not significant; ND, not determined.

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doi:10.1016/j.biocel.2011.06.011

et al., 2007a,b). Notably, urea production solely reflects arginase (ARG) activity. Moreover, the ammonia-eliminating capacity of HepG2 cells via glutamine synthesis is also marginal and even ammonia production has been reported (Harimoto et al., 2005; Nagaki et al., 2001). Furthermore, the HepG2 cells lack CYP enzymes, except fetal isoforms (Donato et al., 2008). The findings for HepG2 are exemplary for most, if not all, human bio-components applied to BALs so far.

In 2002, Gripon et al. reported cell line HepaRG, which approximates primary human hepatocytes (PHHs) in various hepatic functions after culturing for 14 days in HepaRG medium with 2% dimethylsulfoxide (DMSO), preceded by a 14-days proliferation phase without DMSO (Gripon et al., 2002). The HepaRG cultures progress during that 28-day period from a progenitor phenotype into a heterogeneous culture with hepatocyte-like clusters representing 50–55% of the total cell population and neighbouring cells that express biliary epithelial markers (Cerec et al., 2007). A recent microarray analysis showed that HepaRG cells are more related to human liver and PHHs than HepG2 cells (Hart et al., 2010). For most drug-metabolizing genes, including *CYP2B6* and *CYP3A4*, expression levels are associated with the presence of DMSO, possibly via upregulation of nuclear hormone receptors constitutive androstane receptor (CAR) and pregnane X receptor (PXR) (Aninat et al., 2006; Kanebratt and Andersson, 2008). However, DMSO seems ineffective in upregulating the synthesis of blood proteins, like albumin (ALB). Yet, many functions relevant to BAL application and their response to DMSO treatment have not been investigated. Hence, we investigated the effect of DMSO treatment on HepaRG functionality in the context of a BAL, with a focus on nitrogen metabolism. Furthermore, we improved the ureagenesis of HepaRG cells using carbamoyl-glutamate (CG), an analogue of N-acetylglutamate, the physiological allosteric activator of carbamoylphosphate synthetase (CPS), the rate-determining enzyme of the urea cycle under normal physiological conditions (Kim et al., 1972).

2. Materials and methods

2.1. Cell culture

HepaRG cells were kindly provided by Prof. C. Guguen-Guillouzo (INSERM, Rennes). HepaRG cells were cultured in 24-well culture plates (Corning, New York) as described (Gripon et al., 2002). Analyses were conducted on −DMSO and +DMSO cultures, defined as HepaRG cultures 28 days post-seeding, with the last 2 weeks cultured either in absence or presence of 2% DMSO (Sigma, St. Louis), respectively. The effect of CG was tested by adding 1 mM CG (Sigma) either during the test and/or during 5 days preceding the test.

As reference material, small liver samples and PHHs were isolated from liver tissue of patients undergoing partial hepatectomy as described (Hoekstra et al., 2006). The procedure was in accordance with the ethical standards of the institutional committee on human experimentation and after informed consent. The PHHs were seeded at a density of 0.25 million/cm^2 in 24-well plates in HepaRG or WE (Hoekstra et al., 2006) medium. After 4 h attachment, the culture medium was refreshed.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was isolated by using the RNeasy mini kit (Qiagen, Venlo). As a reference, two human liver samples were included in the analyses. cDNA was generated using gene-specific RT primers (Deurholt et al., 2009). Real-time PCR was performed as described (Nibourg et al., 2010). Transcript levels were calculated, normalized for 18S ribosomal RNA, and expressed as a percentage of the mean of the two human liver samples (Hoekstra et al., 2005). Primer sequences and amplicon sizes are depicted in supplemental Table 1.

2.3. Hepatocyte function tests

Hepatocyte function tests were performed on HepaRG cultures and, for comparison, on PHHs at day 1 or 2 post-seeding. Most of the tested liver functions were optimal and stable during that period, however for CYP3A4 activity assays, PHHs were only used as a reference at 1 day post-seeding. After washing the cultures twice with phosphate-buffered saline (PBS, Fresenius Kabi GmbH, Graz) culture medium was replaced by 1 mL of test medium (HepaRG medium with 1.5 mM $^{15}NH_4Cl$ (Sigma), 2.27 mM D-galactose (Sigma), 2 mM L-lactate (Sigma) and 2 mM ornithine hydrochloride (Sigma)). Medium samples were taken after 45 min and 24 h of incubation. For determination of CYP3A4 activity, cultures were incubated with HepaRG medium including 60 μM 3-[(3,4-difluorobenzyl)oxy]-5,5-dimethyl-4-[4-(methylsulfonyl) phenyl]furan-2(5*H*)-one (DFB) (Nicoll-Griffith et al., 2004), kindly provided by Dr. Nicoll-Griffith, Merck Frosst, Canada Limited, Kirkland, and samples were taken after 0.5, 1 and 2 h. Finally, all test cultures were washed twice with PBS and stored at −20 °C for protein determination.

2.4. Biochemical assays

Concentrations of ammonia, urea, apolipoprotein A1 (APOA1), protein and activities of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured in the test samples as reported (Nibourg et al., 2010). Singly and doubly labeled ^{15}N-urea were measured using gas chromatography-mass-spectrometry (Geukers et al., 2005). Amino-acid concentrations were assessed by gradient reversed-phase high-performance liquid chromatography (van Eijk et al., 1988). For CYP3A4 activity, the concentration of 3-hydroxy-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]furan-2(5*H*)-one (DFH), the fluorescent metabolite of DFB, was determined as described (Nicoll-Griffith et al., 2004) after a 2 h-treatment with β-glucuronidase/arylsulfatase (Roche, Basel) (150 Fishman units/mL and 1200 Roy units/mL, respectively) at 37 °C. Production rates were established by calculating the concentration changes in time and were optionally corrected for protein content/well.

2.5. Immunocytochemistry

The cultures were fixed in a 40% methanol (Merck, Darmstadt), 40% acetone (Merck), and 20% water mixture (v/v/v) and stained for GS as described (Deurholt et al., 2009). Similarly, CPS was visualized using a rabbit anti-CPS antibody (1:500 (Charles et al., 1983)) and alkaline phosphatase-labeled goat anti-rabbit IgG 170-6518 (1:200, Bio-Rad, Hercules). ALB was visualized using goat anti-ALB antibody (1:200, Sigma) and alkaline phosphatase-labeled rabbit anti-goat IgG (1:50, Sigma). The reactions were developed with 5-bromo-4-chloro-3′-indolyphosphate p-toluidine and nitro-blue tetrazolium chloride (Roche). In addition, the cultures were evaluated by immunofluorescence for simultaneously staining of CYP3A4 and GS. CYP3A4 was visualized using a rabbit anti-human CYP3A4 antibody (1:100, Fitzgerald Industries International, Acton) and Alexa Fluor 488 (cyan-green)-labeled goat anti-rabbit IgG (1:1000, Molecular Probes, Eugene). GS was visualized using a mouse anti-GS antibody (1:500, Transduction Laboratories, Lexington) and Alexa Fluor 594 (orange-red)-labeled goat anti-mouse IgG (1:1000, Molecular Probes). In addition, cultures were embedded in Vectashield (Vector Laboratories, Burlingame) containing 4,6-diaminidino-2-phenylindole (DAPI) to counterstain DNA. The number of DAPI-stained nuclei was counted manually in ten fields