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ORIGINAL ARTICLE

Stable Overexpression of *Pregnane X Receptor* in HepG2 Cells Increases Its Potential for Bioartificial Liver Application

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To bridge patients with acute liver failure to transplantation or liver regeneration, a bioartificial liver (BAL) is urgently needed. A BAL consists of an extracorporeal bioreactor loaded with a bioactive mass that would preferably be of human origin and display high hepatic functionality, including detoxification. The human hepatoma cell line HepG2 exhibits many hepatic functions, but its detoxification function is low. In this study, we investigated whether stable overexpression of *pregnane X receptor* (*PXR*), a master regulator of diverse detoxification functions in the liver [eg, cytochrome P450 3A (CYP3A) activity], would increase the potential of HepG2 for BAL application. Stable overexpression was achieved by lentiviral expression of the human *PXR* gene, which yielded cell line cBAL119. In monolayer cultures of cBAL119 cells, *PXR* transcript levels increased 29-fold versus HepG2 cells. Upon activation of *PXR* by rifampicin, the messenger RNA levels of *CYP3A4*, *CYP3A5*, and *CYP3A7* increased 49- to 213-fold versus HepG2 cells. According to reporter gene assays with different inducers, the highest increase in *CYP3A4* promoter activity (131-fold) was observed upon induction with rifampicin. Inside BALs, the proliferation rates, as measured by the DNA content, were comparable between the 2 cell lines. The rate of testosterone 6β-hydroxylation, a measure of CYP3A function inside BALs, increased 4-fold in cBAL119 BALs versus HepG2 BALs. Other functions, such as apolipoprotein A1 synthesis, urea synthesis, glucose consumption, and lactate production, remained unchanged or increased. Thus, stable *PXR* overexpression markedly increases the potential of HepG2 for BAL application. ***Liver Transpl 16:1075-1085, 2010.*** © **2010 AASLD.**

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Acute liver failure (ALF) is a severe clinical syndrome with mortality rates as high as 80%. Despite the progress made in supportive care, liver transplantation is the only curative treatment option to date. Liver transplantation is, however, restricted by the limited number of donor organs.[1] To bridge these patients to transplantation, bioartificial liver (BAL) support systems have been developed.[2] BAL systems comprise an extracorporeal bioreactor loaded with hepatocytes that is connected to the patient's circulation. The functionality of these systems obviously relies on the hepatic functionality of the applied cells.

One of the developed BAL systems is the Academic Medical Center bioartificial liver (AMC-BAL), which has shown efficacy in several animal models of ALF and safety in a clinical phase 1 trial.[3] This device and

Abbreviations: ALB, albumin; ALF, acute liver failure; AMC-BAL, Academic Medical Center bioartificial liver; ApoA1, apolipoprotein A-1; ARG1, arginase 1; AST, aspartate aminotransferase; BAL, bioartificial liver; BFC, 7-benzyloxy-4-trifluoromethylcoumarin; CAR, constitutive androstane receptor; cDNA, complementary DNA; CPS, carbamoyl phosphate synthetase; CYP, cytochrome p450; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GS, glutamine synthetase; HFC, 7-hydroxy-4-trifluoromethylcoumarin; HNF4A, hepatocyte nuclear factor 4 alpha; LDH, lactate dehydrogenase; mRNA, messenger RNA; PCR, polymerase chain reaction; PGK, phosphoglycerate kinase; PXR, pregnane X receptor; rRNA, ribosomal RNA; RT, reverse-transcriptase; RT-PCR, reverse-transcriptase polymerase chain reaction; UDCA, ursodeoxycholic acid.

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most other BAL systems rely on primary porcine hepatocytes, but the clinical application of xenogeneic cells is hampered by xenotransplantation-related risks.[4,5] A cell source of human origin should therefore be more suitable for BAL application. A human cell source should exhibit sufficient in vitro hepatic functionality and proliferative capacity. Human liver cell lines are therefore a promising cell source for BAL use.

HepG2 is a human hepatoma cell line derived from a well-differentiated hepatocellular carcinoma of a 15-year-old Caucasian male.[6] This cell line synthesizes a variety of hepatic proteins, such as albumin (ALB) and transferrin.[7] However, HepG2 has a marginal detoxification function because it lacks functional expression of almost all the relevant cytochrome P450s (CYPs).[8] In ALF patients, however, myriad toxic compounds accumulate in the circulation; therefore, a properly functioning detoxification system is a prerequisite for a cell source to be used in a BAL. Accordingly, in a dog model of ALF, treatment with a bioreactor loaded with HepG2 cells overexpressing *CYP3A4* resulted in increased survival in comparison with dogs treated with a bioreactor containing the parental HepG2 cells.[9]

Hepatic detoxification encompasses the biotransformation of hydrophobic toxins into water-soluble substances, which can be secreted into urine or bile. It comprises 4 phases: hepatic uptake, intracellular modification and subsequent conjugation, and secretion. Intracellular modification is typically a hydroxylation reaction carried out by the large family of CYPs. The CYP3A family is the most dominant one at the level of both expression and potency because it metabolizes approximately 50% of all xenobiotics.[10]

Regulation of hepatic detoxification at the molecular level involves a complex interplay between toxic agents (endobiotics and xenobiotics), transcription factors (including nuclear receptors and coreceptors), metabolizing and conjugating enzymes, and import and export transporters. Nuclear receptors, crucial for the regulation of hepatic detoxification, include pregnane X receptor (PXR; nuclear receptor subfamily 1, group I, member 2), hepatocyte nuclear factor 4 alpha (HNF4A), and constitutive androstane receptor (CAR; nuclear receptor subfamily 1, group I, member 3).[11,12] PXR regulates hepatic detoxification at every level. It controls the expression of various transporters (both import and export), CYPs (including the CYP3A family), and conjugation enzymes.[13] To exert its effects, PXR first needs to bind to an activating ligand, such as rifampicin or dexamethasone. Subsequently, this complex translocates to the nucleus, at which it heterodimerizes with the retinoic X receptor and then interacts with the regulatory sequences of its target genes.[11]

Rifampicin is a very potent activator of PXR in both human hepatocytes and HepG2 cells. Using transient transfection assays, several studies have shown an increase in *CYP3A4* promoter activity in HepG2 cells upon the addition of rifampicin. However, the extent of *CYP3A4* promoter activation in these studies has varied from 5- to 20-fold. This is possibly due to differences in cell culture conditions or the HepG2 cell line used.[14-16]

In stably transfected HepG2 cells overexpressing *PXR* (the DPX-2 cell line), rifampicin increased *CYP3A4* promoter activity 35-fold and CYP3A4 metabolic activity 3- to 5-fold in comparison with uninduced cells.[16,17] However, both studies lacked a comparison with the ancestral HepG2 cell line, so the effect of *PXR* overexpression on the detoxification function remains unknown. In addition, other hepatic functions and the proliferative capacity of the cells were not examined in these studies. Consequently, the previous results do not unambiguously indicate the potency of *PXR* overexpression in HepG2 cells for BAL application.

Therefore, we stably overexpressed the human *PXR* gene in HepG2 cells by lentiviral transduction and produced the new cell line cBAL119. We compared the functionality of cBAL119 with that of the parental HepG2 cells, and by characterizing its hepatic functionality, metabolism, and proliferative capacity in monolayer and laboratory models of the AMC-BAL, we studied its potential for BAL application.[18]

MATERIALS AND METHODS

Generation of cBAL119

The complementary DNA (cDNA) of the human *PXR* gene, kindly provided by Dr. Jean Marc Pascussie (University of Montpellier, Montpellier, France), was introduced into HepG2 cells by lentiviral transduction. The lentiviral vector backbone was described as prrlcpptpgkgfppressin by van Til et al.[19] A puromycin resistance gene, preceded by a *phosphoglycerate kinase* (*PGK*) promoter, was inserted into the *Eco* RV site, and the *green fluorescent protein* gene was replaced by the human *PXR* gene. In this way, a lentiviral vector, pBAL119, was generated with 2 PGK promoters controlling the expression of both the *PXR* and puromycin resistance gene. The construct was confirmed by DNA sequence analysis.

The lentivirus was produced essentially as previously described.[20] In brief, HEK293T cells were transiently transfected with the SuperFect transfection reagent (Qiagen) with a third-generation lentiviral vector system, including pBAL119. The virus-containing supernatant was collected 24, 48, and 72 hours after transfection, filtered through 0.45-μm Millipore filters, and immediately transferred to HepG2 cells in a subconfluent state in 6-well plates in a 1:1 lentivirus preparation: culture medium ratio. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and a penicillin and streptomycin mix (100 U/mL penicillin and 100 μg/mL streptomycin; all reagents from Lonza). Three days after transduction, the cells were passaged to a T75 flask, and 20 μg/mL puromycin was included in the culture

medium to select for HepG2 cells with an integrated lentiviral construct; this yielded the polyclonal cell line cBAL119.

Monolayer Culture and Cell Isolation

The cBAL119 and parental HepG2 cells were cultured in DMEM culture medium at 37°C in a humidified atmosphere (95% air and 5% CO_2). For large-scale expansion, the cell lines were cultured in Corning CellSTACK culture chambers (5-stacked). The culture medium was refreshed every 3 days. Cells were detached with a 1:1 mixture of trypsin (0.25%)/ethylene diamine tetraacetic acid (0.03%; Lonza) and Accumax (Innovative Cell Technologies; vol/vol), centrifuged at 50*g* for 5 minutes, and washed twice with ice-cold DMEM culture medium and once with cold Williams' E (WE) culture medium (Lonza) supplemented with 4% heat-inactivated FBS (vol/vol), 2 mM L-glutamine, 20 mU/mL insulin (Novo Nordisk), 2 mM ornithine hydrochloride (Sigma-Aldrich), and a penicillin and streptomycin mix (100 U/mL penicillin and 100 μg/mL streptomycin).

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA was isolated from cBAL119 and parental HepG2 cells cultured to confluence in a monolayer (6-well plates) with the RNeasy mini kit (Qiagen). As a reference, 2 different human liver samples were included in the analyses. First-strand cDNA was generated from 1 μg of total RNA with a reverse-transcriptase (RT) primer mix comprising 11 pmol of the 18S ribosomal RNA (rRNA) antisense primer and 25 pmol of gene-specific RT primers targeting the genes listed in Table 1 with 134 U of Superscript III (Invitrogen) in a total volume of 25 μL at 50°C for 1 hour. The reaction mix was heated to 70°C for 15 minutes and then cooled to 4°C. The resulting gene-specific RT reaction mixes were column-purified to remove gene-specific primers with a QIAquick polymerase chain reaction (PCR) purification kit (Qiagen) and were brought to a volume of 100 μL.

Real-time PCR with a LightCycler 480 SYBR Green kit (Roche) was performed on 1 μL of the RT reaction mix according to the manufacturer's instructions. Primer sequences and amplicon sizes are depicted in Table 1. The thermal cycling profile of the touchdown PCR was as follows: denaturation at 95°C for 3 minutes, 41 cycles of 94°C for 1 s, primer annealing at 68°C for 7 s with a 0.5°C/cycle decrease until 63°C, and extension for 20 s at 72°C.

Transcript levels were calculated as described, subsequently normalized for 18S rRNA, and expressed as percentages of the mean starting levels of 2 human liver samples.[21]

CYP3A4 Promoter Activity Analysis

HepG2 and cBAL119 cells were seeded in 24-well plates at 45% confluence. After 2 days, the cells were

TABLE 1. Primer Sequences Used in the RT-PCR Analyses and Resulting Amplicon Sizes

Gene	RT Primer	Sense Primer	Antisense Primer	Amplicon Size (bp)
18S rRNA	CGAACCTCCGACTTTCGTTT	TTCGGAACTGAGGCCATGAT	CGAACCTCCGACTTTCGTTCT	151
PXR	TCTGGTCCTCGATGGGCAAGTC	GAGAGCGGCATGAAGAAGGAGA	CATGTGGGGCAGCAGGAGAAG	420
HNF4A	CACTCCAACCCCGCCCCTC	CCGGGTGTCCATACGCATCCT	CAGGTTGTCAATCTTGGCC	321
CAR	GCTCTTCTTGCTCCTTACTC	CGTCATGGCCAGTAGGGAAG	CATGCCAGCATCTAAGCACT	232
CYP3A4	CCCGTGAGAAGCAGAGGA	AGCTTAGGAGGACTTCTTCAACC	AGCCAAATCTACCTCCTCACACT	313
CYP3A5	GGTACCATCTCTTGAATCCACC	TGACCCAAAGTACTGGACAG	TGAAGAAGTCCTTGCGTGTC	240
CYP3A7	AGCCAAATCTACTTCCCCAGCAC	ATTACGCTTTGGAGGACTTCTTCT	CGTCTTCATTTCAGGGTTCTATTT	182
ALB	ACTTCCAGAGCTGAAAAGCATGGTC	TGAGCAGCTTGGAGAGTACA	GTTCAGGACCACGGATAGAT	189
CPS	CAGCTGTCCTCCGAATCAC	GAAGGGGCCCGAGAAGTAGAA	CTCAACCGGGGCCAGGAAAAC	445
ARG1	TGTGATTACCCTCCCGAGCAAGTC	TTGGCAAGGTGATGGAAGAAACA	CCTCCCGAGCAAGTCCGAAACAA	305
GS	TTGGCAGAGGGGCGACGAT	GCCTGCTTGTATGCTGGAGTC	GGCGCTACGATTGGCTACAC	420

NOTE: Primers are indicated from 5′ to 3′.

transfected with the plasmids pRL-TK (0.27 μg/well) and CYP3A4-XREM-Luc (5.5 μg/well), which were kindly provided by Dr. Richard Kim (University of Western Ontario, London, Canada), with Lipofectamine 2000 (Invitrogen).[15] After transfection, the cells were cultured in 500 μL of WE culture medium, which included 1 μL of inducers at different concentrations [ie, rifampicin (Sigma), dexamethasone (Centrafarm), and ursodeoxycholic acid (UDCA)] or dimethyl sulfoxide (DMSO) as a vehicle control (for all, n = 3). After 16 hours, the medium, including additions, was refreshed, and 24 hours later, the relative *CYP3A4* promoter activity was assessed with a dual luciferase reporter assay kit (Promega).

Bioreactor

We used the laboratory-scale AMC-BAL bioreactor, which is a 55× down-scaled bioreactor of the third-generation AMC-BAL with an internal volume of 10 mL.[22] The general configuration of the bioreactor consists of a polycarbonate housing containing a 3-dimensional, nonwoven, hydrophilic polyester matrix circularly wound around a polycarbonate core. Between the matrix layers, hydrophobic polypropylene gas capillaries are situated in a parallel fashion. The ends of these capillaries are embedded in polyurethane resin and are fitted with gas inlet and outlet caps.[18]

Bioreactor Culture

Bioreactors were loaded with 9-mL cell suspensions containing 1.6 mL of cell pellets of HepG2 or cBAL119 cells (for both, n = 6). Loaded bioreactors were placed in a temperature-controlled culture cabinet (37°C) and oxygenated with a mixture of 40% O_2, 55% N_2, and 5% CO_2 (vol/vol). Bioreactors were subjected to a 340° transverse and longitudinal rotation for 3 hours to ensure optimal cell attachment and an even cell distribution. After this attachment period, dead and unattached cells were removed by a flush of 40 mL of the culture medium; afterward, the bioreactors were continuously perfused with 100 mL of a recirculating BAL culture medium [WE culture medium with 10% FBS (vol/vol) instead of 4% FBS and 1 μM dexamethasone without phenol red] supplemented with 10 μM rifampicin at 5 mL/minute. Every day, the culture medium was completely refreshed.

Determination of the Bioactive Mass

To determine the bioactive mass, we investigated samples of the cell suspensions loaded into the bioreactors for the cell pellet volume after centrifugation, cell counts, aspartate aminotransferase (AST) content, lactate dehydrogenase (LDH) content, total protein content, and total DNA content (for all, n = 4 per cell line). The cell pellet volume was determined after centrifugation at 50*g* for 3 minutes. Cell counts were performed with a Bürker Bright line cytometer (Optik Labor). Every cell suspension sample was counted twice, and means were used for calculations. For intracellular AST and LDH contents, a 100-μL cell suspension was lysed by the addition of 900 μL of 2% Triton X (Sigma-Aldrich) and subsequent vortexing. These lysates were diluted 100× and measured for the AST and LDH contents as described previously.[23] Total protein and DNA contents were measured in cell pellets obtained from 100-μL cell suspensions by centrifugation at 50*g* for 3 minutes. Cell pellets were washed in phosphate-buffered saline and recentrifuged twice before pellets were incubated overnight in 1 mL of 0.2 M NaOH (Merck) at 37°C. In these lysates, the total protein content was measured with the Coomassie Brilliant Blue G-250 staining kit (Bio-Rad), and the total DNA content was measured with the Hoechst 33258 reagent as described by Downs et al.[24] To measure proliferation inside the BAL, the total DNA content was also analyzed in HepG2 BALs and cBAL119 BALs (for both, n = 5) after a 4-day culture period. Therefore, both ends of all bioreactors were removed after 4 days of culture; subsequently, the total BAL contents were removed from the housing and shaken in 35 mL of 0.2 M NaOH at 37° for 3 days. After 3 days, the lysate was harvested, and the extraction was repeated twice for 3 consecutive days with fresh NaOH. The DNA and protein contents were determined in all 3 lysates, and the total DNA and protein contents per BAL were then calculated. The last lysate did not contain any detectable protein or DNA.

Bioreactor Function Tests

Function tests were performed with a test medium composed of the BAL culture medium supplemented with 125 μM testosterone (Fluka) or 100 μM 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 2 mM L-lactate (Sigma), 2.75 mM D-galactose (Sigma-Aldrich), and 5 mM NH_4Cl (Merck). Rifampicin was omitted from the test medium because it would have disturbed the measurement of urea. Bioreactors were first flushed with 30 mL of the test medium, and this was followed by a 3-hour period of recirculation with 41 mL of the test medium. Samples (1 mL) were taken at 30, 60, 120, and 180 minutes of recirculation and analyzed for concentrations of urea, glucose, and lactate and for activities of AST and LDH, as previously described.[23,25] 6β-Hydroxytestosterone production was measured with a high-performance liquid chromatograph coupled to a mass spectrometer. The conversion of BFC to 7-hydroxy-4-trifluoromethylcoumarin (HFC) was measured fluorometrically at the excitation wavelength of 410 nm and the emission wavelength of 510 nm after deconjugation of the samples with β-glucuronidase/arylsulfatase (150 Fisherman units/1200 Roy units; Roche) at 37°C for 2 hours and termination of the reaction in 0.25 M tris-hydroxymethylaminomethane/60% acetonitrile (vol/vol).[26] Apolipoprotein A-1 (ApoA1) production was assessed by an enzyme-linked immunosorbent assay

with a polyclonal rabbit anti-human ApoA1 (Calbiochem) in a 1:500 dilution as the primary antibody, a monoclonal mouse anti-human ApoA1 (Calbiochem) in a 1:500 dilution as the secondary antibody, and a polyclonal rabbit anti-mouse immunoglobulin G horseradish peroxidase (Dako) in a 1:2000 dilution as the tertiary antibody. The reaction was developed with 3,3′-diaminobenzidine as a chromogen. Function parameter rates were determined by the calculation of the changes in the concentration in the medium per hour per gram of protein loaded into the BALs.

Statistical Analysis

Repeated measurement analyses of variance tests were used to compare differences between the 2 bioreactor groups (HepG2 and cBAL119) over the 4-day culture period. Paired Student *t* tests were performed to compare daily differences within 1 experimental group. Unpaired Student *t* tests were performed to compare differences between 2 experimental groups per day. Factor correction was used to correct for between-session variation in multisession experiments.[27] SPSS 16.0.1 (SPSS, Inc., Chicago, IL) was used for statistical analysis. Prism version 4.01 (GraphPad Prism, Inc., San Diego, CA) was used for graphical presentation of the data. Data are expressed as means and standard deviations. Significance was reached at $P < 0.05$.

RESULTS

Transcript Levels of *PXR* and Target Genes Are Increased in cBAL119 Cells

A cell line named cBAL119 was generated by transduction of HepG2 cells with a lentiviral construct carrying the human *PXR* gene as well as the puromycin resistance gene. Selection for puromycin resistance generated more than 50 colonies, which together yielded the cBAL119 cell line.

Subsequently, transcription levels of various hepatic genes were determined in monolayer cultures of HepG2 and cBAL119 in the absence and presence of rifampicin (Table 2). The expression of *PXR* in uninduced cBAL119 cells was increased 29-fold versus the parental HepG2 cells and was up to 583% of the in vivo level; this indicated the establishment of *PXR* overexpression. As expected, the addition of rifampicin had no effect on transcript levels of *PXR* in the 2 cell lines. The messenger RNA (mRNA) levels of 2 other relevant nuclear hormone receptors (*HNF4A* and *CAR*) were not changed upon overexpression or activation of *PXR*. The mRNA level of *HNF4A* was 484% of the in vivo level, which was already high in HepG2 cells, whereas the *CAR* transcript level was less than 1% of the in vivo level.

As for the PXR target genes of the CYP3A family, we observed significant 18- and 7-fold increases in *CYP3A5* and *CYP3A7* mRNA levels, respectively, in

TABLE 2. Transcript Levels of Several Hepatic Genes in HepG2 Cells and cBAL119 Cells

	PXR (%)	*HNF4A* (%)	*CAR* (%)	*CYP3A4* (%)	*CYP3A5* (%)	*CYP3A7* (%)	*ALB* (%)	*CPS* (%)	*ARG1* (%)	*GS* (%)
HepG2 without rifampicin	20.2 ± 8.2	484.2 ± 181.3	0.5 ± 0.2	0.006 ± 0.004	1.3 ± 0.3	3.6 ± 3.1	10.5 ± 8.1	3.8 ± 1.1	11.2 ± 5.1	1564.2 ± 623.8
HepG2 with rifampicin	28.6 ± 4.4	664.7 ± 198.1	0.6 ± 0.4	0.022 ± 0.010*	4.3 ± 2.3*	6.5 ± 2.6	17.8 ± 15.3	5.1 ± 1.1	9.7 ± 4.8*	919.8 ± 200.2
cBAL119 without rifampicin	583.0 ± 363.3*,†	389.4 ± 70.3†	0.8 ± 0.4	0.012 ± 0.004	23.5 ± 6.7*,†	23.7 ± 6.2*,†	55.9 ± 22.9*	3.4 ± 1.6	11.2 ± 3.7	925.4 ± 580.4
cBAL119 with rifampicin	535.4 ± 210.9*,†	419.8 ± 257.6	0.6 ± 0.4	0.926 ± 0.402*,†,‡	64.3 ± 42.9*,†	766.3 ± 724.1	35.4 ± 23.9	1.6 ± 0.1*,†	11.7 ± 11.7	341.0 ± 79.9*,†

NOTE: Data were obtained in 4 separate experiments and were corrected for between-session variation as described. Values are expressed as percentages of human in vivo levels and as means and standard deviations.

*$P < 0.05$ for the denoted group versus HepG2 without rifampicin.

†$P < 0.05$ for the denoted group versus HepG2 with rifampicin.

‡$P < 0.05$ for the denoted group versus cBAL119 without rifampicin.

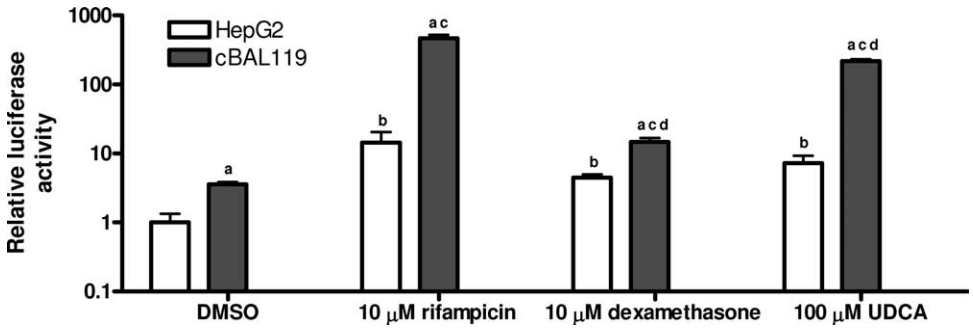

Figure 1. *CYP3A4* promoter activity of HepG2 cells and cBAL119 cells according to a luciferase reporter gene assay. *CYP3A4* promoter activity was normalized to uninduced HepG2 cells. Significance was determined as follows: [a]$P < 0.05$ for cBAL119 versus HepG2 with the same inducer, [b]$P < 0.05$ for HepG2 with an inducer versus HepG2 with DMSO (vehicle), [c]$P < 0.05$ for cBAL119 with an inducer versus cBAL119 with DMSO, and [d]$P < 0.05$ for cBAL119 with dexamethasone or UDCA versus cBAL119 with rifampicin.

TABLE 3. Quantification of the Bioactive Mass in HepG2 Cells and cBAL119 Cells

	Cell Pellet Volume ($\mu L/10^6$ cells)	AST ($U/10^6$ cells)	LDH ($U/10^6$ cells)	Protein ($\mu g/10^6$ cells)	DNA ($\mu g/10^6$ cells)
HepG2	4.20 ± 1.04	1.77 ± 0.48	5.28 ± 0.62	67.1 ± 20.9	226.5 ± 77.7
cBAL119	3.76 ± 0.90	1.69 ± 0.84	4.75 ± 1.63	52.8 ± 22.4	177.4 ± 85.6

NOTE: No significant differences were observed between HepG2 cells and cBAL119 cells. Values are expressed as means and standard deviations.

cBAL119 cells to approximately 23% to 24% of the in vivo levels. Upon the addition of rifampicin, transcription levels of *CYP3A5* and *CYP3A7* further increased in cBAL119 to 64% and 766% of the in vivo levels. The transcript level of *CYP3A4* was significantly (42-fold) increased in cBAL119 versus HepG2 only after the addition of rifampicin. However, the transcription level remained low in induced cBAL119 cells (<1% of the in vivo level).

Differential Effects on Transcript Levels of Other Hepatic Genes in cBAL119 Cells

The transcript levels of other hepatic genes considered to be relevant for BAL application were also investigated. These included ALB as a synthetic marker, 2 urea cycle enzymes [carbamoyl phosphate synthetase (CPS) and arginase 1 (ARG1)], and glutamine synthetase (GS), which, in addition to the urea cycle, is relevant for the elimination of ammonia. Unexpectedly, the *ALB* transcript levels were increased 5-fold (significant) in uninduced cBAL119 cells to 55% of the in vivo levels. The transcript levels of *CPS*, *ARG1*, and *GS* were unchanged in cBAL119 versus HepG2. The mRNA levels of *ARG1* and *CPS* were both low in uninduced cBAL119 cells (11% and 3.4% of the in vivo levels, respectively), whereas the *GS* transcript level was 925%. The addition of rifampicin resulted in a trend of diminishing transcript levels of *ALB*, *CPS*, and *GS* in cBAL119.

CYP3A4 Promoter Activity Is Increased in cBAL119 Cells, and Rifampicin Is the Most Potent Inducer

To test the induction capacity of different inducers, *CYP3A4* promoter activity was assessed in both HepG2 cells and cBAL119 cells with a luciferase reporter gene assay. The results shown were obtained from 1 representative experiment (Fig. 1). Without induction, *CYP3A4* promoter activity in cBAL119 cells was significantly increased (3.6-fold) versus HepG2 cells.

CYP3A4 promoter activity was significantly higher in cBAL119 cells versus HepG2 cells upon the addition of the PXR inducers rifampicin, dexamethasone, and UDCA. The effects were most pronounced with rifampicin. In cBAL119 cells, the addition of rifampicin significantly increased *CYP3A4* promoter activity in comparison with uninduced cBAL119 cells (130-fold) and induced HepG2 cells (32-fold).

These results show that *PXR* overexpression increased both basal and inducible *CYP3A4* promoter activity, and rifampicin was the most potent inducer.

HepG2 Cells and cBAL119 Cells Proliferate Inside the BAL During Culture

To determine the bioactive mass, several parameters were analyzed in 4 isolates of both HepG2 and cBAL119 cells (Table 3). The cell pellet volume and DNA and protein levels per million cells were not significantly different in cBAL119 cells versus HepG2

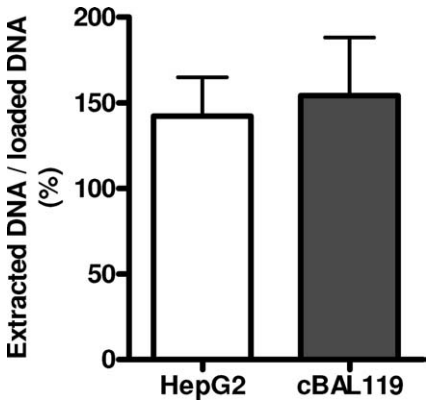

Figure 2. Proliferation during the BAL culture as determined by increases in the total DNA content. Values are expressed as means and standard deviations. Differences were not significant.

cells, and this indicated no effect of *PXR* overexpression on the cell volume and DNA and protein content per cell. We observed a high correlation between DNA and protein levels within different isolates of HepG2 ($r = 0.98$) or cBAL119 ($r = 1.00$).

Subsequently, proliferation of both cell lines during BAL culture was calculated by the measurement of the total DNA content at the time of loading and after 4 days of culture. The DNA content increased 1.4-fold for HepG2 BALs and 1.5-fold for cBAL119 BALs in 4 days of culture (Fig. 2). The difference between the 2 cell lines was not significant, and this indicated no differences in proliferation capacity between HepG2 cells and cBAL119 cells in the BALs under the given conditions.

Cellular Integrity Is Maintained during BAL Culture in Both HepG2 BALs and cBAL119 BALs

First, we assessed the total AST and LDH contents per million cells in samples of the cell suspensions that were loaded into the BALs (Table 3). Then, to analyze cell integrity during BAL culture, we determined the AST and LDH release in both BAL groups. Enzyme levels did not significantly differ between HepG2 cells and cBAL119 cells (Fig. 3A,B).

With respect to the total AST and LDH contents, the release rates were very low for both groups during the whole culture period. In cBAL119 BALs, the maximum AST release rate was 2.23 ± 1.32 U hour^{-1} g of protein^{-1}, and the maximum LDH release rate was 5.59 ± 1.65 U hour^{-1} g of protein^{-1}. This corresponded to a negligible loss of approximately 0.3% to 0.4% of the total cell mass per BAL per day. In addition, enzyme leakage was generally highest on day 1, and this possibly reflected cell damage as a result of the isolation procedure.

6β-Hydroxytestosterone Production Rate Is Increased in cBAL119 BALs

We determined the production rate of 6β-hydroxytestosterone as a parameter for detoxification function in

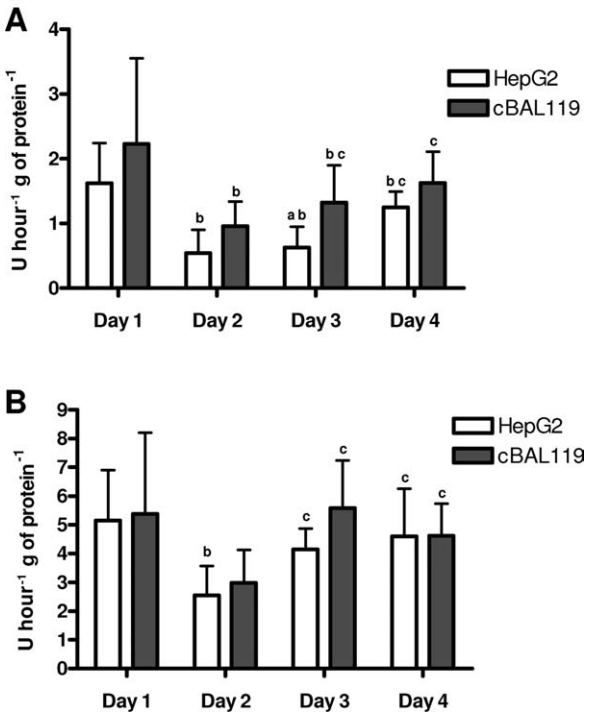

Figure 3. Cellular integrity analysis in HepG2 BALs and cBAL119 BALs: (A) AST and (B) LDH release. Values are expressed as means and standard deviations. Significance was determined as follows: $^{a}P < 0.05$ for cBAL119 versus HepG2 on the same day, $^{b}P < 0.05$ for the denoted day versus day 1 for the same cell line, and $^{c}P < 0.05$ for the denoted day versus day 2 for the same cell line.

both HepG2 BALs and cBAL119 BALs. On any tested day over the 4-day culture period, cBAL119 BALs produced significantly more 6β-hydroxytestosterone than HepG2 BALs (Fig. 4). On day 1, the 6β-hydroxytestosterone production rate in cBAL119 BALs reached 0.95 μmol hour^{-1} g of protein^{-1}; this was 1.6% of the level produced by primary human hepatocytes in monolayer cultures (data not shown). In addition, the largest difference between HepG2 BALs and cBAL119 BALs was also observed on day 1 with a 3.8-fold higher production rate in cBAL119 BALs. From day 1 to 2, the 6β-hydroxytestosterone production rate in cBAL119 BALs decreased, and a trend of an increase in the production rate in cBAL119 BALs started on day 2. In HepG2 BALs, the 6β-hydroxytestosterone production rate remained stable throughout the culture period.

As an additional parameter for detoxification, we analyzed the conversion rate of BFC to HFC in both cell lines on the second day of BAL culture. Although there was a trend toward a higher production rate in cBAL119 BALs versus HepG2 BALs (46.6 ± 19.8 versus 56.2 ± 7.5 nmol hour^{-1} g of protein^{-1}), this difference was not significant ($P = 0.146$).

Functions Other Than Detoxification Differ Marginally Between cBAL119 and HepG2 BALs

As a marker for synthetic function, we determined the ApoA1 production of the HepG2 and cBAL119 BALs.

Over the whole culture period, the ApoA1 production rate in cBAL119 BALs was not significantly different from that in HepG2 BALs (Fig. 5A). However, in comparison with the rate in HepG2 BALs, the ApoA1 production rate in cBAL119 BALs was significantly higher on days 1 and 3. In both HepG2 BALs and cBAL119 BALs, the ApoA1 production rate significantly decreased during culture.

The urea production rate in cBAL119 BALs was not significantly different from that in HepG2 BALs (Fig. 5B). In cBAL119 BALs, the urea production rate decreased significantly from day 2 to day 3 and increased again from day 3 to day 4.

During BAL culture, the glucose consumption rate increased approximately 2-fold between days 1 and 4 (significant) in both HepG2 BALs and cBAL119 BALs, and there were no significant differences between the 2 BAL groups (Fig. 5C). Between the 2 BAL groups, the lactate production rate was similar, and it increased during culture (Fig. 5D). For both groups, this increase was approximately 2-fold between days 1 and 4.

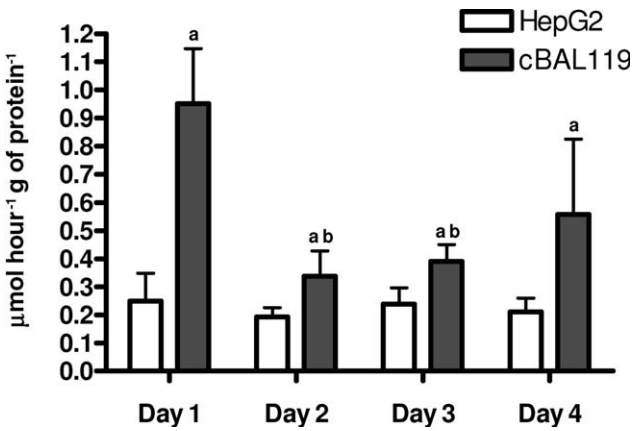

Figure 4. 6β-Hydroxytestosterone production in HepG2 BALs and cBAL119 BALs. Values are expressed as means and standard deviations. Significance was determined as follows: $^{a}P < 0.05$ for cBAL119 versus HepG2 BAL on the same day and $^{b}P < 0.05$ for the denoted day versus day 1 for the same cell line.

DISCUSSION

In the present study, we created the cell line cBAL119 by stably overexpressing the human *PXR* gene in HepG2 cells. We compared the cBAL119 cells with the parental HepG2 cells on various levels in both monolayer cultures and BAL cultures. Here we provide evidence that stable overexpression of *PXR* in HepG2 cells increases the potential of these cells for BAL application. This is based on the following observations in cBAL119: (1) an increase in the expression levels of several important hepatic genes involved in detoxification; (2) an increase in *CYP3A4* promoter activity; (3) an increase in detoxification activity, as shown by an increase in the rate of 6β-hydroxylation of testosterone; and (4) maintenance of the proliferation capacity and liver cell functionality, as tested by DNA analysis and several general cellular and hepatic functions.

Because of the xenogeneic risks related to the use of animal hepatocytes in BALs, the success of the clinical application of BAL treatment is highly dependent on the availability of a human cell line possessing high hepatic functionality. An effective hepatic detoxification system is one of the prerequisites of such a cell line. This is supported by the observations of Wang et al.,[9] who showed that treatment with a BAL filled with HepG2 cells overexpressing *CYP3A4* increased the survival of dogs with ALF. Besides

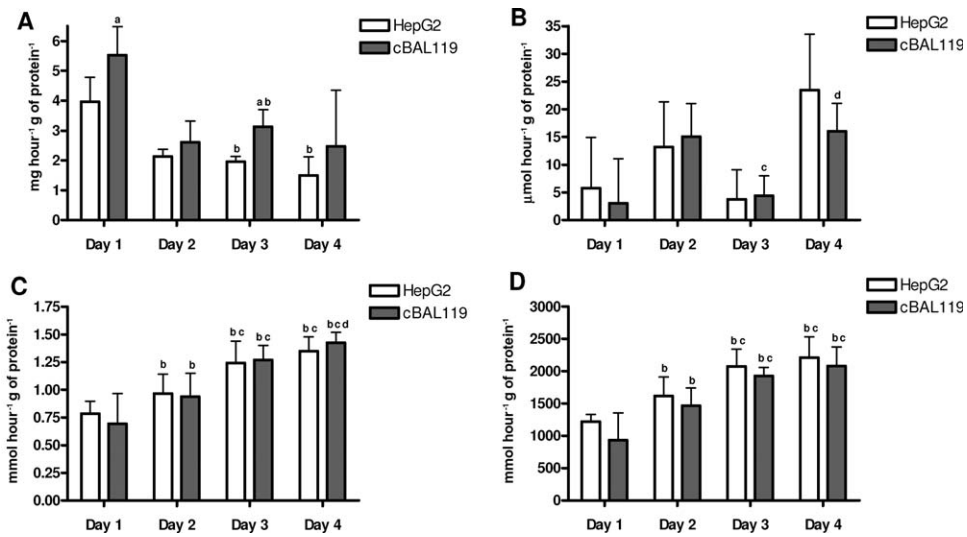

Figure 5. Nondetoxification functions of HepG2 BALs and cBAL119 BALs: (A) ApoA1 production, (B) urea production, (C) glucose consumption, and (D) lactate production. Values are expressed as means and standard deviations. Significance was determined as follows: $^{a}P < 0.05$ for cBAL119 versus HepG2 on the same day, $^{b}P < 0.05$ for the denoted day versus day 1 for the same cell line, $^{c}P < 0.05$ for the denoted day versus day 2 for the same cell line, and $^{d}P < 0.05$ for the denoted day versus day 3 for the same cell line.

CYP3A4 activity, however, effective detoxification of endotoxins and exotoxins also depends on import and subsequent conjugation and canalicular export systems. For example, during the detoxification of high concentrations of acetaminophen, the initial hydroxylation by CYPs generates the very toxic *N*-acetyl-*P*-benzoquinone imine, and it is only by the subsequent conjugation to glutathione and excretion that the molecule is detoxified. Therefore, overexpressing *PXR* as a regulator of detoxification on every level (import, hydroxylation, conjugation, and export) may be a more promising approach to increasing detoxification function. We used a third-generation lentiviral vector; the genes encoding the structural lentiviral proteins are expressed only in the vector-producing cells and not in the target cells. Hence, the risks of possible replication and infection of patients treated with cBAL119 BALs are negligible.[20,28]

We showed a 29-fold higher expression of *PXR* in cBAL119 cells versus HepG2 cells, and this is indicative of successful overexpression. As a result, the cBAL119 cells showed increased basal and inducible transcript levels of *CYP3A4*, *CYP3A5*, and *CYP3A7*. The transcription level of *CYP3A4* remained relatively low, and this may not inconceivably be related to the low expression of *CAR*. However, the homology between CYP3A4 and other family members (CYP3A5 and CYP3A7) is almost 90%, and as a result, CYPs within one family share many substrates.[29] Therefore, the activity of CYP3A5 and CYP3A7 may compensate for the lack of CYP3A4 activity. In addition, CYP3A7 is principally a fetal CYP3A member, and this explains the high transcript levels versus the adult liver.[30]

CYP3A4 promoter activity in basal (ie, uninduced) cBAL119 cells was increased 3.6-fold versus uninduced HepG2 cells. Tirona et al.[15] reported a 1.4-fold increase in this respect. Upon induction by rifampicin, *CYP3A4* promoter activity in cBAL119 cells increased further (130-fold with respect to the level of uninduced cBAL119 cells and 465-fold with respect to the level of uninduced HepG2 cells). In contrast, Tirona et al. reported a 43-fold increase in *CYP3A4* promoter activity in their *PXR*-transfected cells upon induction with rifampicin. In another study, Luo et al.[14] found an increase in *CYP3A4* promoter activity of approximately 20-fold upon the induction of rifampicin in their *PXR*-transfected HepG2 cells. Thus, the *CYP3A4* promoter activity in cBAL119 cells is markedly higher than the *CYP3A4* promoter activity found in both of these studies.

In addition, HepG2 cells transiently overexpressing *PXR* are not suitable for BAL application because a stable cell line is a prerequisite for BAL application. In this respect, only one study reported HepG2 cells stably overexpressing the *PXR* gene (DPX-2 cells). In this study, *CYP3A4* promoter activity in rifampicin-induced DPX-2 cells was increased 30-fold versus uninduced DPX-2 cells.[17] This increase was also markedly less than the 130-fold increase that we observed.

Detoxification on a functional level, as measured by the 6β-hydroxylation rate of testosterone, was significantly higher in cBAL119 BALs versus HepG2 BALs on every day of culture. On day 1, this difference was most pronounced (almost 4-fold). Although this increase is substantial, it is only a fraction of the increase in the *PXR* transcript levels (19-fold) or *CYP3A4* promoter activity (32-fold). It cannot be excluded that other factors, such as posttranscriptional regulation of CYPs (eg, CYP oxidoreductase activity) and transport processes that are not controlled by PXR, are rate-limiting. After day 1 of culture, testosterone hydroxylation in cBAL119 BALs declined. On day 2, metabolic rates were at their minimum, and from day 2 to day 4, we observed a trend toward an increase in testosterone hydroxylation. This pattern may be associated with the organization and hepatic differentiation of the cells. The cells probably still showed the monolayer phenotype at the protein level on day 1, but the isolation procedure and resulting loss of cell-cell contacts led to diminished CYP3A expression on day 2. Greuet et al.[31] previously showed that cell-cell contacts are a prerequisite for high CYP3A4 expression and activity in primary human hepatocytes. The increase in testosterone hydroxylation, starting on day 2, may be explained by a gradual restoration of cell-cell contacts and/or by cell proliferation. Interestingly, the absence of these trends in HepG2 cells, even though the growth rates of cBAL119 and HepG2 were similar, suggests that the effect of cell organization on the 6β-hydroxylation of testosterone is PXR-mediated. The 6β-hydroxylation rate of testosterone in cBAL119 BALs reached approximately 1.6% of the rate of primary human hepatocytes in monolayer cultures. Although this is not substantial, it is unknown whether this amount is sufficient in terms of treating ALF patients with cBAL119 BALs. To test this hypothesis, cBAL119 BALs will have to be compared with HepG2 BALs in an animal model of ALF.

As an additional parameter for CYP activity, we also studied BFC conversion rates in BALs loaded with both cell lines on the second day of culture. The conversion of BFC to HFC is catalyzed predominantly by CYP1A1, CYP1A2, and CYP2B1 and only to a lesser extent by CYP3A4.[26,32,33] Our data show a trend toward an increased conversion rate in cBAL119 BALs, but we did not find a significant difference between the 2 cell lines. This is possibly related to a lack of up-regulation of *CYP1A1*, *CYP1A2*, and *CYP2B1* in cBAL119 cells. *CYP2B1* is not a target gene of PXR in humans, and this supports the hypothesis.[13] In contrast, CYP1A1 and CYP1A2 have been described as target genes for PXR in humans; however, we did not measure higher CYP1A2 mRNA levels in cBAL119 cells versus HepG2 cells (data not shown). This could also underlie the lack of difference in HFC production between the 2 cell lines.

The cBAL119 and HepG2 cells exhibited similar growth rates, urea production, lactate production, and glucose consumption as well as similar levels of AST and LDH leakage. However, the transcript levels of *ALB* and the ApoA1 production rate both

significantly increased in cBAL119 cells; this indicates that hepatic synthetic function is increased in cBAL119. This is in agreement with recent experiments demonstrating that the activity of PXR is not restricted to detoxification. PXR also exerts its activity on energy metabolism and binds to promoters of genes implicated in other processes such as apoptosis and electron transport; indeed, the *APOA1* gene has previously been identified as a target of PXR.[34-36]

Unfortunately, the urea production rate was low in both cell lines when they were cultured in the AMC-BAL; it was approximately 10% of the rate of a BAL filled with primary porcine hepatocytes. Moreover, Mavri-Damelin et al.[37] demonstrated that urea production in HepG2 cells does not originate from exogenously added ammonia because the urea cycle is nonfunctional in HepG2 cells. Instead, urea production is mainly due to intramitochondrial arginase II activity.[37] Our data support these findings because ammonia elimination was negligible in both HepG2 and cBAL119 BALs (data not shown), and the urea cycle genes *CPS* and *ARG1* were expressed at very low levels in both cell lines.

Because the clinical application of liver support systems relying on cell lines is dependent on the stability of these cell lines, it would be interesting to investigate whether the functionality of cBAL119 BALs will further improve during prolonged culture. In general, our data show that the cBAL119 BALs demonstrated the highest hepatic functionality on day 1, a decrease on day 2, and subsequently a regain of function on days 3 and 4. As discussed, cellular organization–related differentiation could underlie this phenomenon, and it is therefore not inconceivable that this gain in functionality will continue during prolonged culture. Additional studies of cBAL119 BALs will be necessary to address this question.

In conclusion, our data demonstrate that stable overexpression of *PXR* in HepG2 cells improves their detoxification function with conservation of other hepatic functions and proliferation capacity. Because detoxification plays a central role in the treatment of ALF, *PXR* overexpression in HepG2 cells increases the potential of this cell line for BAL application. However, improved detoxification will not necessarily translate into increased survival when these cells are tested in an animal model of ALF. In addition, further research is needed to improve the ammonia elimination and urea production capacity of the cBAL119 cells or alternatively to investigate the possibility of combining a cBAL119 BAL with an additional ammonia detoxification device. Subsequently, such a system should show proof of principle in an animal model of ALF before a phase 1-2a study in patients with ALF can be started.

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