Human liver cell lines for the AMC-bioartificial liver

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CHAPTER 2

Proliferative human cell sources applied as biocomponent
in bioartificial livers: a review


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Ruurdtje Hoekstra
ABSTRACT

Background: Bioartificial livers (BALs) are urgently needed to bridge severe liver failure patients to liver transplantation or liver regeneration. When based on primary hepatocytes, their efficacy has been shown in animal experiments and their safety was confirmed in clinical trials. However, a proliferative human cell source with therapeutic functionality is needed to secure availability and move BAL application forward.

Aim: This review compares the performance of BALs based on proliferative human biocomponents and on primary hepatocytes.

Methods: This review evaluates relevant studies identified by searching the MED-LINE database until July 2011 and some of our own unpublished data.

Results and conclusions: All the discussed hepatocyte-like biocomponents show deficiencies in their hepatic functionality compared to primary hepatocytes, particularly functions occurring late in liver development. Nonetheless, the HepaRG, HepG2-GS-CYP3A4 and mesenchymal stem cells show efficacy in a statistically well-powered animal model of acute liver failure, when applied in a BAL device. Various methods to gain higher functionality of BALs, including genetic modification, the usage of combinatory cell sources, and improvement of culture methods have scarcely been applied, but may further pave the path for BAL application. Clinical implementation of a BAL based on a human proliferative biocomponent is still several years away.
INTRODUCTION

Acute liver failure (ALF) and acute-on-chronic liver failure (ACLF) are severe syndromes with mortality rates as high as 80%. In this review the term severe liver failure (SLF) will be used for both ALF and ACLF. Clinically, the syndromes present as a severe impairment of liver function with hepatocellular necrosis, leading to hepatic encephalopathy (HE), systemic inflammation, and multi-organ failure (MOF). Notably, the pathological processes underlying this cascade of events are only partly understood.

Liver transplantation (LT) is often the only cure for SLF patients, increasing the survival rates to over 80%. However, LT is limited by the scarcity of donor organs. In the Western world, about 20-25% of all liver patients (including SLF patients) on the waiting list for LT die while waiting for a donor liver, and this number is increasing. Consequently, there is an urgent need to bridge SLF patients to LT or to liver regeneration. Bioartificial extracorporeal liver support devices can fulfill this need.

Bioartificial livers (BALs) use a biocomponent, e.g. hepatocytes in extracorporeal bioreactors that are perfused with the patient's blood or plasma. These systems can theoretically replace all crucial liver functions, and are therefore a more promising option for liver replacement therapy than non-biological systems that only rely on detoxification. BAL devices with various configurations have been developed; Flat plate systems for 2D cultures and systems that support cell organization in 3D relying on semipermeable hollow fibers, scaffolds, and encapsulated cells. All these devices vary in their oxygenation, cell-plasma contact, shear stress, complexity, costs and potential to upscaling.

In addition to the device configuration and culture process, successful BAL therapy relies on the hepatic functionality of the biocomponent. There is robust evidence that accumulating levels of ammonia play an important role in the progression of the disease. In addition, several other neurotoxins may act synergistically with ammonia in the progression of HE. Therefore, a BAL should adequately eliminate ammonia and other neurotoxins. Ammonia can be eliminated either by urea production or by fixation into amino acids (predominantly by glutamine synthetase (GS) activity) of which the first route is preferred as urea is an end-product. Other neurotoxins are predominantly detoxified by components associated with drug metabolism, i.e. phase 1 (particularly cytochrome P450 (CYP) 3A4) and phase 2 detoxification proteins. In SLF-patients, the lack of other functions such as lactate elimination, glucose homeostasis, and the synthesis of blood proteins including pro- and anti-coagulative factors can lead to serious complications such as acidosis, hypoglycemia, diminished suppression of inflammation, and thrombosis or bleeding. Therefore, a BAL should effectively replace these functions as well. In addition, anti-inflammatory properties are probably useful for a BAL, to suppress the systemic inflammation, developing in SLF.
Table 1. Overview of proliferative human cell sources applied in bioartificial livers.

For colored table, see page 212-217.

Tones of grey indicate the potential for BAL application as indicated by in vitro functionality or efficacy in animal studies:
white: no data available.
darkest tone grey: in vitro functionality < 5% of primary hepatocytes, or not effective in animal study;
middle tone grey: in vitro functionality > 10% of primary hepatocytes, or proven efficacy in animal study
lightest tone grey: inconclusive

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<td>Pos Alb, AFP, transferrin Detectable 7-ethoxycoumarin metabolism, level inconclusive</td>
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<td>Clonal derivative of HepG2, selected for strong contact inhibition, high production of albumin and AFP and growth in glucose deficient medium</td>
<td>Diverse devices, most often in ELAD</td>
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# Proliferative human cell sources applied as biocomponent in bioartificial livers: a review

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<td>Iwahori et al. Hepatol. 2003; Kosuge et al. Liver Int. 2007</td>
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<td>Darnell et al. Drug Metab Dispos. 2011; Nibourg et al. Unpublished data.</td>
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Ring et al. Tissue Eng Part C. 2010  
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<td>Akiyama et al. Int J Mol Med. 2004</td>
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| Lactate production and glucose consumption | Two animal studies. The first study in monkeys with D-galactosamine intoxication with inconclusive results. The second study in pigs with ALF, ongoing. | | Kobayashi et al. J Artif Org. 2005  
| Production | Lactate production converting to consumption and glucose consumption | Werner et al. Ann New York Acad Sci. 1999  
| Pos, but substantial decline in time | Lactate production and glucose consumption | | |
| Detectable, level inconclusive | | | |

Proliferative human cell sources applied as biocomponent in bioartificial livers: a review
Table 1. Continued

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<th>Genetically modified cells</th>
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<td>Perfusion bioraector withi porous scaffold</td>
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* although the ATCC datasheet states that HepG2 does not form tumours in SCID mice, several investigators use HepG2 cells to induce tumour formation in SCID mice (Sun, 2004 459 /id; Lu, 2003 460 /id).

** Ureagenesis can be positive without a functional urea cycle.

Abbreviations: AFP, alpha fetoprotein; Alb, albumin; ApoA1, apolipoprotein A-1; Arg, arginase; ALF, acute liver failure; AMC-BAL, Academic Medical Center-BAL; CYP, cytochrome P450; ELAD, extracorporeal liver assist device; elim, elimination; GS, glutamine synthetase; HE, hepatic encephalopathy;
HNF4, hepatocyte nuclear factor 4; hTERT, human telomere reverse transcriptase; neg, negative; IL-1, interleukine 1; OTC, ornithine transcarbamoylase; pos, positive; pRb, Retinoblastoma protein; prod, production; PH, primary hepatocyte; PXR, pregnane X receptor; revers imm PHH, reversibly immortalized primary human hepatocytes; SV40, simian virus 40.
Proliferative human cell sources applied as biocomponent in bioartificial livers: a review

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<th>Tones of grey</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>No OTC expression</td>
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<td>Saito et al. World J Gastroenterol. 2006</td>
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<td>Lactate production and glucose consumption</td>
<td>Lactate production and glucose consumption</td>
<td>Nibourg et al. Liver Transplant. 2010</td>
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<td>One animal study (n=9) in rats with D-galactosamine intoxication. No positive effects on survival. Significant reduction IL-6 levels.</td>
<td>One animal study (n=9) in rats with D-galactosamine intoxication. No positive effects on survival. Significant reduction IL-6 levels.</td>
<td>Shinoda et al. J Surg Res. 2007</td>
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<td>One animal study (n=7-8) in dogs with total liver ischemia: pos effect on survival time and diazepam metabolism</td>
<td>One animal study (n=7-8) in dogs with total liver ischemia: pos effect on survival time and diazepam metabolism</td>
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<td>Two animal studies. The first study in pigs with total liver ischemia (n=8-9): positive effect on survival time. The second study in dogs with total liver ischemia (n=5-7): no effect on survival time</td>
<td>Two animal studies. The first study in pigs with total liver ischemia (n=8-9): positive effect on survival time. The second study in dogs with total liver ischemia (n=5-7): no effect on survival time</td>
<td>Enosawa et al. Cell Transplant. 2000; Enosawa et al. Cell Transplant. 2001; Enosawa et al. Transplant Proc. 2001; Enosawa et al. Cell Transplant. 2006</td>
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Tones of grey indicate the potential for BAL application as indicated by in vitro functionality or efficacy in animal studies. White: no data available. Darkest tone grey: in vitro functionality < 5% of primary hepatocytes, or not effective in animal study. Lightest tone grey: inconclusive. Middle tone grey: in vitro functionality > 10% of primary hepatocytes, or proven efficacy in animal study. *Although the ATCC datasheet states that HepG2 does not form tumours in SCID mice, several investigators use HepG2 cells to induce tumour formation in SCID mice. **Ureagenesis can be positive without a functional urea cycle. Abbreviations: AFP, alpha fetoprotein; Alb, albumin; ApoA1, apolipoprotein A-1; Arg, arginase; ALF, acute liver failure; AMC-BAL, Academic Medical Center-BAL; CYP, cytochrome P450; ELAD extracorporeal liver assist device; elim, elimination; GS, glutamine synthetase; HE, hepatic encephalopathy; HNF4, hepatocyte nuclear factor 4; hTERT, human telomere reverse transcriptase; neg, negative; IL-1, interleukine 1; OTC, ornithine transcarbamoylase; pos, positive; pRb, Retinoblastoma protein; prod, production; PH, primary hepatocyte; PXR, pregnane X receptor; revers imm PHH, reversibly immortalized primary human hepatocytes; SV40, simian virus 40.
Primary human hepatocytes (PHHs) are the ideal cell source for BAL application and there have been case reports and a phase I study describing their safe use in ALF patients. However, due to limited availability, high variability and complex logistics the chances of a PHH-BAL eventually reaching the clinic are negligible. Alternatively, BALs based on primary porcine hepatocytes (PPHs) have proven efficacious in animal models of ALF. However, the use of animal-derived cells is compromised by xenotransplantation-related risks. Therefore, BAL devices can only be clinically successful when based on a functional human biocomponent that is readily available in large quantities, \textit{i.e.} that proliferates \textit{in vitro}.

Some previously published reviews discuss the potential of proliferative human cell sources for BAL application. However, none of these reviews focus on those cell sources that have actually been applied in BAL devices. These include hepatoma derived cell lines, \textit{in vitro} immortalized hepatocytes, and immature cells such as fetal liver cells or stem cells. In addition, all these cell sources can be genetically modified to increase their performance, and combinations of different cell sources can be used (Table 1).

Therefore, the purpose of this review is to compare all proliferative human cell sources that have been applied in BAL systems and evaluate their potential. To this end, we searched the MED-LINE database on key words related BAL systems and human cells, and reviewed the published literature until July 2011. In addition, we included unpublished data from our own experiments.

\textbf{ASSESMNENT AND COMPARISON OF CELLULAR FUNCTIONALITY AND BIOARTIFICIAL LIVER SYSTEMS}

Comparing the functionality of human bio-components in BAL systems entails some difficulties, since the performance of such BAL systems does not only depend on the biocomponent, but also on the BAL configuration, culture and/or test conditions (medium flow rate, static or dynamic culture). Therefore, proper comparison of the bio-components in different BAL devices is only possible when a study has incorporated a positive control group, \textit{i.e.} freshly isolated primary mammalian hepatocytes (PHs) cultured similarly in the same device. In the case that these data are not available, a functional comparison was made upon the basis of literature data, and we feel that conclusions based upon these data must be drawn with reserve.
HEPATOMA CELL LINES

Hepatoma cell lines are derived from hepatoblastomas or hepatocellular carcinomas. The most frequently used hepatoma cell line for BAL application is the HepG2 cell line. Nyberg et al. compared BALs with HepG2 cells or primary rat hepatocytes (PRHs). In this study the detoxification function of PRH-BALs clearly outperformed that of HepG2-BALs, as demonstrated by an over 233-fold higher oxidation rate and a 30-fold higher glucuronidation rate. Ammonia elimination is highly variable between different HepG2-BALs ranging from 64% the rate of elimination of PPH-BALs to ammonia production. Carbohydrate metabolism of HepG2 cells deviates from that of PHHs. HepG2-BALs produce lactate and consume glucose, whereas PHH-BALs consume lactate and produce glucose. Consumption of glucose and the subsequent conversion into lactate despite sufficient oxygen supply, is a well-known feature of tumor cells, known as the Warburg effect and may affect BAL culture and even stimulate lactic acidosis in SLF patients. On the other hand, the synthetic functions of HepG2-BALs are markedly high with low variability between different BAL devices. In Nyberg’s study, the rate of albumin synthesis reached an exemplary 74% of the rate in PRH-BALs. Others have reported synthesis of fibrinogen, ApoA1 and alpha-fetoprotein (AFP) in their HepG2-BALs. However, it should be noted that AFP is typically an immature hepatocyte marker, and therefore has limited value. Only one group evaluated a HepG2-BAL in an animal study. Acetaminophen-overdosed rabbits were treated with alginate-encapsulated HepG2-beads. This treatment resulted in some hemodynamic improvement, but ammonia levels, bilirubin levels and prothrombin time did not improve, and survival studies were not included.

To increase the functionality of HepG2, Kelly et al. selected clones for strong contact inhibition (exit of the cell cycle short after reaching confluence), high albumin production, high AFP production, and the ability to grow in glucose-deprived medium, yielding cell line C3A. In a study by Carraro et al. C3A-BALs demonstrated CYP1A2, CYP2B6, and CYP2E1 activity, but this study lacks comparison with PH-BALs. Both ammonia elimination and production are reported in different C3A-BALs, possibly related to the differences in BAL systems and culture environment. Urea production in C3A cells is – at least on monolayer and similarly to HepG2 cells – a result of Arginase 2 (Arg2) activity, and not of a functional urea cycle. C3A-BALs are capable of gluconeogenesis, as reported by Ellis et al. However, in other studies C3A-BALs consumed glucose and also produced lactate. Synthetic functions of C3A-BALs are comparable to PH-BALs, including the production of albumin. Despite the lack of convincing in vitro data, C3A-BALs have been tested in animals and particularly in patients. In 1991 Kelly et al. reported on the treatment of anhepatic dogs with a hollow fiber BAL loaded with C3A cells, named the extracorporeal liver assist device (ELAD). Three dogs were treated with this BAL system and 3 control dogs received supportive care. One dog treated with the ELAD lived 12.5 hours, whereas all other 5 lived 3 to 5 hours. In addition, BAL treatment
did not improve blood ammonia levels. In an uncontrolled follow up study with 3 dogs with acetaminophen-induced ALF, two survived the experiment. These small and uncontrolled studies do not justify any conclusions on efficacy of therapy. Nevertheless, patient studies with the ELAD were initiated. After a clinical phase I study, efficacy of the ELAD was tested in a randomized controlled pilot study in 17 patients with potentially recoverable ALF and 7 listed for LT. In both groups, no effect of on survival, HE grade and blood ammonia levels was observed. After these studies, the ELAD was modified (ultrafiltrate instead of blood perfusion through the ELAD, increased fiber permeability and cell mass) and proved safe in a new phase I clinical trial. A phase II controlled clinical trial performed in China followed, of which no results have been published in peer-reviewed journals yet. Currently, a follow-up phase II clinical trial has started in the US.

Next to HepG2 and C3A, a few other hepatoma cell lines have been applied in BAL systems. Yamashita et al. compared the Huh7 cell line with the HepG2 cell line and PPHs, cultured in a polyurethane foam/spheroid BAL system. In this study, ammonia elimination and albumin production in the Huh7-BAL were 38% and 50%, respectively, of the PPH-BALs, but the HepG2-BALs outperformed the Huh7-BALs.

Three other hepatoma cell lines, named FLC-4, FLC-5, and FLC-7, were tested in radial flow BALs. The expression levels of albumin and coagulation factors were <10% and 80% of the in vivo level, respectively. None of the cell lines expressed the complete set of genes involved in ammonium or drug metabolism. The FLC-4 radial flow bioreactor was further evaluated in a pig model of α-amanitin and lipopolysaccharide induced ALF. Both the two control pigs died in this study, whereas all three treated pigs survived. Again however, the small numbers of experiments do not justify any conclusions on the efficacy of this FLC-4-BAL. The FLC-5-BAL and FLC-7-BALs were only tested in uncontrolled in vitro studies. The FLC-5-BAL expressed CYP3A4 and showed functional CYP3A4 activity which increased substantially upon induction by rifampicin. The FL-7-BAL produced albumin and AFP, and consumed glucose. As mentioned above however, AFP production is a characteristic of fetal liver cells and not of mature hepatocytes, and glucose consumption (instead of production) does not add to its hepatospecificity.

Recently, the hepatoma cell line HepaRG has been evaluated for BAL application. The HepaRG cell line is a bipotent hepatocyte progenitor cell line that differentiates in vitro into two distinct types of cells: hepatocyte-like cells, and biliary-like cells upon reaching confluence in monolayer cultures, and further differentiation can be realized by culturing in the presence of 2% dimethyl sulfoxide (DMSO), a modulator of chromatin structure, for two weeks. This way, the HepaRG cell line exhibits exceptionally high detoxification functionality in vitro, thereby providing a valuable tool for drug metabolism studies. In a study by Darnell et al., HepaRG cells were cultured in a three-dimensional (3D) multi-compartment capillary membrane bioreactor. The cells were proliferated for 2 weeks without DMSO, followed by a differentiation phase for
2 weeks in the presence of 2% DMSO. During the whole culture period, this HepaRG-BAL produced lactate and albumin. After differentiation, activities of CYP1A1/2, CYP2B6, CYP2C9, and CYP3A4 were tested both 3-days and several weeks after omission of DMSO, and were found to be similar, except for CYP2B6, which increased. In this study, no PH-BAL was included and bioactive mass was not quantified, rendering quantitative interpretation of these results difficult. In our lab, we cultured the HepaRG cell line in the AMC-BAL according to a modified culture protocol without DMSO. After 14 culture days of BAL culture, CYP3A4 activity was 31% of the rate of PHHs cultures on monolayer. Ammonia elimination and ureagenesis were 144% and 23% of the rates of PPH-BALs, respectively. A $^{15}$N ammonia loading experiment revealed that 27% of all formed urea was produced by a functioning urea cycle. In addition, the HepaRG-BAL demonstrated liver-specific carbohydrate metabolism by consumption of lactate (148% the rate of PPH-BALs). Finally, synthetic functionality of the HepaRG-BAL was also high as ApoA1 production reached 192% the rate of PHHs on monolayer. Subsequently, this HepaRG-BAL was tested in rats with ALF, induced by total liver ischemia (n=5-6). Treatment with HepaRG-BALs resulted in a significantly increased survival time of ~50%, and a delayed increase of HE, renal failure, and blood ammonia levels.

In conclusion, hepatoma cell lines show a large variety in their performance, particularly related to detoxification, nitrogen and carbohydrate metabolism. Of these cell lines the HepaRG cell line shows most promise, but still has to prove efficacy in a clinical trial. However, testing of the C3A cell line in the ELAD system has advanced most progressively by entering its third clinical phase II trial.

**IN VITRO IMMORTALIZED CELLS**

Immortalization implies overcoming normally occurring cell cycle arrests *in vitro*. Progression of the cell cycle from the G1 phase to the S phase is controlled by both positive (e.g. cyclins, cyclin-dependent kinases, transcription factor E2) any negative regulators (e.g. tumor suppressor proteins p53, p21, p16, and the retinoblastoma protein (pRb)), and by telomere length. Strategies to immortalize human liver cells include manipulation of the expression of cell-cycle controlling genes and of *human telomerase reverse transcriptase (hTERT)*, the catalytic subunit of telomerase that restores telomere length after each cell division.

In a study from Werner et al., immortalized PHHs were generated by downregulating p53 and pRb, and by overexpressing the *EF2* and *cyclin D1* genes. This new cell line HepZ, was cultured in a bioreactor composed of microporous gelatin microcarriers. The HepZ-BAL consumed glutamine and glucose, and produced ammonia during the 10 days of culture. Lactate production was seen the first 7 days, but switched to a minor consumption during the last 3 days of culture. In addition, the HepZ-BAL displayed CYP activity by metabolizing lidocaine.
Chapter 2

Unfortunately, this study was not PH-BAL controlled. Nonetheless, ammonia production obviously is an undesirable feature for a BAL.

In our lab, we generated the cBAL111 cell line by overexpressing \( hTERT \) in human fetal liver cells (FLCs).\(^5\) cBAL111 cells were tested in the AMC-BAL at two different loading densities.\(^6\) The high density cBAL111-BALs eliminated ammonia up to a level of 49% of PPH-BALs. As urea production only reached ~1% of the level of PPH-BALs and glutamine concentrations increased after ammonia loading, ammonia elimination was probably predominantly the result of GS activity. Albumin synthesis and detoxification function, as tested by lidocaine elimination, were low in cBAL111-BALs, with rates of 6% and 0.1% relative to PPH-BALs respectively. Furthermore, cBAL111-BALs were unable to prolong survival in a rat model of ALF (data not published).

Akiyama et al. evaluated the OUMS-29 cells and OUMS-29/H11 cells in a radial flow bioreactor.\(^6\) The OUMS-29 cell line was generated by transfecting FLCs with the gene encoding the simian virus 40 large T antigen which perturbs p53 and pRb function.\(^6\) The OUMS-29-BALs produced albumin at a rate of ~10% the rate of PRH-BALs in Nyberg’s study.\(^2\) In addition, no CYP3A4 protein was detected in OUMS-29-BALs.

Kobayashi et al. reported on the efficacy of human hepatocytes reversibly immortalized by overexpressing the \( hTERT \) under cre/loxP control.\(^6\) This elegant model allows expansion of the cells until sufficient bioactive mass, followed by inhibition of expansion by deletion of the \( hTERT \) gene through cre-recombinase activity, with concomitant differentiation and reduction of potential tumorigenesis. In a monkey model of \( D \)-galactosamine-induced ALF, empty BAL treated monkeys died within 5 days, whereas monkeys treated with BALs loaded with immortalized hepatocytes or PPHs (positive control) survived for over 14 days.\(^6\) Unfortunately however, the number of animals used and the statistical analysis are lacking in this study. In a follow-up study Kobayashi et al. reported a significant improvement of survival and biochemical parameters in pigs with ALF when treated with a BAL loaded with reversibly immortalized hepatocytes.\(^6\) Again however, detailed outcomes and statistical analysis were not provided.

In conclusion, the immortalized cell lines that have been applied in BALs so far display insufficient hepatic functionality, or lack necessary data on efficacy in animal models of SLF.

**FETAL LIVER CELLS AND STEM CELLS**

A third group of BAL biocomponents are immature human cells, including FLCs and stem cells from embryonic, mesenchymal and hepatic origin.

FLCs, in general harvested by digestion of fetal livers with collagenase followed by low-speed centrifugation, display sufficient proliferative capacity *in vitro*, as they can undergo 58 population doublings before entering growth arrest.\(^6\) We compared FLC-based AMC-BALs
cultured for 7 days with PHH-BALs and PPH-BALs. Although both FLC-BALs and PPH-BALs produced the same amount of urea, FLC-BALs produced ammonia, in contrast to PPH-BALs. Albumin production was comparable in all three groups, and lidocaine elimination was over highest in FLC-BALs. Furthermore, FLC-BALs produced lactate, and switched to glucose consumption after 3 days of culture, probably reflecting dedifferentiation. Two other studies also reported lactate production in FLC-BALs. In addition, these studies reported albumin synthesis and CYP3A4 and CYP3A7 expression, although no quantitative comparison was made with mature hepatocytes.

Small hepatocytes (SHs), originally first identified in rats, are committed hepatocyte progenitor cells that can proliferate in vitro for over 3 months, and have the capacity to differentiate into mature hepatocytes in vitro. In an uncontrolled study by Pavlic et al., SHs were tested in a BAL prototype comprising a rotary cell culture system and displayed detoxification function by both eliminating diazepam and oxazepam, and metabolizing ethanol. In addition, SH-BALs produced urea at approximately the same rate as the PRH-BALs in Nyberg’s study, and albumin production reached twice the PRH-BAL rate. Two follow-up studies by Wurm et al. confirmed the previously observed functionality. In one of these studies, ureagenesis could even be increased 3-fold upon a challenge with 1 mM ammonia. On the other hand, lactate was produced and morphine was not metabolized.

Another study by Fonsato et al. exploited human liver stem cells (HLSCs) that initially express mesenchymal and embryonic stem cell markers, and no oval cell markers. BAL culture enhanced the differentiation of HLSCs substantially. They produced high amounts of hepatocyte growth factor (HGF), lost expression of stem cell markers and displayed CYP activity a rate comparable to PHH-BALs as observed by metabolism of ethoxy-4-trifluoromethylcoumarin. Furthermore, HLSC-BALs produced urea and albumin up to a rate of 51% and 69% of PHH-BALs, respectively. Notably, mesenchymal stem cells (MSCs) (see below) were cultured in the same BAL and MSC-BALs produced urea and albumin at a rate of 15% and 17%, respectively, of the PHH-BALs, which demonstrates that MSCs have less potential than HLSCs to differentiate into hepatocyte-like cells under this protocol.

Next to functional hepatic replacement, controlling inflammation can provide additive therapeutic support in SLF treatment. MSCs exert an immunomodulatory effect by inhibiting the function of various immune cells in vitro. In a study by Parekkadan et al., MSC-BALs significantly increased the survival ratio (71% versus 14%) of rats with D-galactosamine-induced ALF (n=7) compared to fibroblast-BALs and empty BALs. As treatment with MSC-conditioned medium resulted in significant improvement of survival as well, the effect is probably chemokine-related. In a study from Kazemnejad et al., human bone marrow-derived MSCs were cultured in a three-dimensional nanofibrous scaffold bioreactor and differentiated into hepatocyte-like cells using HGF, dexamethasone, and the cytokine oncostatin M. In contrast to undifferentiated MSCs, MSC-bioreactors produced urea, aspartate aminotransferase,
alanine aminotransferase, and transferrin. Unfortunately, no PHH-BAL group was included as positive control, and concentrations of the abovementioned products were presented without a given volume, rendering quantitative comparison with PH-BALs impossible.

Recently, Miki et al. reported on human embryonic stem cells (hESC) that were cultured in a hollow fiber-based 3D perfusion bioreactor and differentiated into hepatocytes using a growth factor cocktail protocol. They demonstrated that the induced 3D culture outperformed the 2D and uninduced controls in albumin and urea production, as well as the expression of several hepatic genes. However, the rate of albumin production in the induced hESC-BAL was less than 1% of the rate of the PRH-BAL in Nyberg’s study.

In conclusion, several types of immature cells offer interesting possibilities for BAL application. SHs and HLSCs both exhibit high hepatic functionality in vitro, although efficacy in animal models needs to be proven. MSCs have shown efficacy in an inflammatory model of ALF and might offer an interesting cell source for hepatic differentiation. FLCs are no likely candidates for BAL application, as they produce ammonia and dedifferentiate after a few days of culture.

**FUNCTIONALY MODIFIED CELLS**

The functionality of a potential BAL biocomponent can be increased by overexpressing structural hepatic genes or hepatic transcription factors. In addition, genes can be introduced with supportive characteristics, *e.g.* with anti-inflammatory or anti-apoptotic effects. An additional advantage of overexpressing a single gene, is that it allows an assessment of contribution of that particular function in BAL therapy by comparing the therapeutic effects of modified cell-BALs and their parental cell-BALs.

**Modified hepatoma cell lines**

As described above, HepG2-BALs display a low and inconsistent ammonia elimination, varying from ammonia production to reasonably high ammonia elimination. However, in most studies ammonia elimination and urea cycle activity are low. Therefore, Enosawa et al. overexpressed the *glutamine synthetase (GS)* gene, yielding the cell line HepG2-GS. Although ammonia elimination through ureagenesis is preferable, as indicated previously, GS overexpression may increase the ammonia elimination. The HepG2-GS cell line was cultured in a radial flow bioreactor and the HepG2-GS-BALs eliminated ammonia at 15% the rate of PPH-BALs. Next, this HepG2-GS-BAL was evaluated in ALF pigs with liver ischemia (*n*=8-9). Mean survival was significantly higher in the HepG2-GS-BAL group compared to the empty BAL treated group.

The same group further aimed to improve the HepG2-GS cell line by additionally overexpressing the *CYP3A4* gene, yielding the HepG2-GS-CYP3A4 cell line. The HepG2-
GS-CYP3A4-BALs eliminated ammonia at a rate of 36% of PPH-BALs. Next, treatment of ALF-dogs (induced by total liver ischemia and an additional overdose of diazepam) with a HepG2-GS-CYP3A4-BAL \((n=8)\) resulted in a significantly longer survival time (42.7 hours) than treatment with a HepG2-GS-BAL (22.1 hours; \(n=7\)) or an empty BAL (31 hours; \(n=5\)). In addition, significantly more diazepam was metabolized in the HepG2-GS-CYP3A4-BAL group compared to both the control groups. These results underline the importance of detoxification function for a BAL. Interestingly however, this study reported no increased survival time by HepG2-GS-BAL treatment in contrast to a previous report. Possibly this inconsistency is related to differences in the ALF models used. Nonetheless, further evaluation of the HepG2-GS cells and the HepG2-GS-CYP3A4 cells is necessary to investigate their efficacy, and the requirement of GS and CYP3A4 activity for BAL application.

By stably overexpressing the *pregnane X receptor (PXR)*, a key regulator of drug metabolism, in HepG2 cells, we undertook a different approach to overcome the low detoxification function of HepG2 cells. In this new cell line, cBAL119, mRNA levels of CYP3A4, CYP3A5, and CYP3A7 increased 7- to 42-fold and CYP3A4 activity as measured by 6β-hydroxylation of testosterone was increased 4-fold versus HepG2. Unfortunately, CYP3A4 activity still reached only about 1 percent of that of PPHs cultured on monolayer. In addition, cBAL119 BALs did not eliminate ammonia nor produced urea. Therefore, cBAL119 most likely displays insufficient hepatic functionality for BAL application.

To increase ammonia elimination and urea production in HepG2 cells, Coward et al., overexpressed the genes encoding the urea cycle enzymes ornithine transcarbamylase (OTC) and arginase 1 (both limiting urea cycle activity in HepG2 cells), yielding the cell line HepG2-DT. In the HepG2-DT-BAL, incorporation of \(^{15}\)N labeled ammonia into urea increased 10-fold, reaching 20% the level of PH monolayer cultures. The HepG2-DT-BALs produced the same amount of albumin as HepG2-BALs in this study. Although these results are promising, further functional improvements (detoxification, carbohydrate metabolism) are probably necessary to make HepG2 cells suitable for BAL application.

**Modified in vitro immortalized cells**

Shinoda et al. overexpressed the *interleukin 1 receptor antagonist (IL-1Ra)* gene in the conditionally immortalized cell line TTNT. IL-1Ra is a competitive inhibitor of IL-1, a very potent pro-inflammatory cytokine. IL1-Ra producing TTNT cells were cultured in a flat-plate BAL. Treatment of D-galactosamine-induced ALF rats \((n=9-14)\) did not significantly reduce hepatocellular leakage, nor improve the survival ratio at 28 days after treatment. In contrast, both treatment with a PRH-BAL overexpressing *IL-1Ra* as well as venous infusion of the IL-1Ra protein did increase the survival ratio in ALF rats.

The OUMS-29 cell line (described above) displayed low expression levels of *hepatic nuclear factor (HNF) 4A*, a major regulator of hepatic differentiation and no CYP3A4 expression.
Therefore, the OUMS-29/H-11 cell line was generated by overexpressing HNF4α.\textsuperscript{60, 84} In contrast to OUMS29-BALs, CYP3A4 protein was produced by OUMS-29/H-11-BALs; however, the authors state that the level was “much lower” than in human liver. OUMS-29/HNF-11-BALs also demonstrated 6β-hydroxylation of testosterone as a marker for CYP3A4 function in a late phase of the BAL culture; however, the activity was not quantified. OUMS-29/H-11-BALs have not been tested for efficacy in an animal model of SLF.

In conclusion, clear effects of overexpression of genes on BAL biocomponents are reported, and may certainly add to their functionality, if basic hepatic activity is present.

**COMBINATIONS OF CELLS**

Heterotypic interactions between hepatocytes and non-parenchymal liver cells are known to modulate cell growth and increase or stabilize hepatic functionality.\textsuperscript{85} Therefore attempts have been made to co-culture hepatocytes in a BAL with supporting cells, like MSCs, hepatic stellate cells/cell lines, or endothelial cells/cell lines. Some of these BALs will probably not reach clinical application, as they currently rely on animal cells and/or PHs. Nevertheless, we will discuss four examples to illustrate their potential.

In a study by Saito et al., the hepatoma cell line FLC-5 was co-cultured in a radial flow bioreactor with the mouse endothelial cell line M1 and the mouse hepatic stellate cell line A7.\textsuperscript{86} Compared to a FLC-5-BAL, expression levels of albumin and HNF4A decreased significantly in the FLC-5/M1/A7-BAL, suggesting a dedifferentiation and/or suppressing effect of M1/A7 co-culture. Although the FLC-5/M1/A7-BAL, in contrast to the FLC-5-BAL, produced urea, gene expression analysis of the urea cycle genes revealed no expression of OTC. Therefore the observed urea production is not the result of urea cycle activity, but most likely of non-hepatocyte specific arginase 2 activity.

Endo et al. evaluated the functionality of a double-compartment BAL in which the PCTL-MDR cell line, a rabbit proximal tubule (kidney) cell line overexpressing the apical efflux transporter multidrug resistance protein 1 (MDR1), was co-cultured with the HepG2 or the HepG2-CYP3A4 cell lines.\textsuperscript{87} The rationale for the use of the PCTL-MDR cells was that these cells are capable of transporting the drugs digoxin and doxorubicin solely in a basolateral-to-apical direction.\textsuperscript{88} The PCTL-MDR cells were cultured on a tight monolayer on one side of the double-compartment BAL, and HepG2-GS or HepG2-GS-CYP3A4 cells were cultured on the opposite side. Although they do not present data, the authors state that testosterone hydroxylation was “very low” in het PCTL-MDR/HepG2-GS-BAL, whereas it was “almost equal to isolated human hepatocytes” in the PCTL-MDR/HepG2-GS-CYP3A4-BAL. Another study with the same PCTL-MDR/HepG2-GS-CYP3A4-BAL reported lidocaine metabolism and selective transport from the outer to the inner compartment (not quantified).\textsuperscript{89} In this study,
the lactate concentration increased on the PCTL-MDR side, whereas the concentration on the HepG2-GS-CYP3A4 side remained constant. The ammonia concentration decreased on the HepG2-GS-CYP3A4 side and remained constant on the PCRL-MDR side. Nonetheless, the ammonia elimination rate only reached ~1% of PPHs on monolayer.\textsuperscript{90}

In a study by Yagi et al., PRHs were co-cultured with MSCs to enhance hepatic functionality and introduce anti-inflammatory activity.\textsuperscript{91} On monolayer PRH-MSCs co-cultures showed increased albumin secretion and CYP1A1 activity compared to PRH cultures. In addition, treatment of  D\textsubscript{-}galactosamine-induced ALF rats with a PRH-MSC-BAL (n=9) increased their 20-day survival ratio substantially compared to fibroblast/PRH BAL treatment (n=10). Separate MSC-BAL and PPH-BAL groups were not included in this study, which would have been interesting to isolate the effect of co-culture. Nonetheless, this study underlines the potential of MSCs to stabilize and increase hepatic functionality of the co-cultured cells.

In a recent study by Carraro et al., HLSCs were co-cultured with hepatic stellate cells in a perfused bioreactor.\textsuperscript{92} The co-culture secreted albumin at a maximally 1.4-fold increased rate. No other liver-specific functions were assessed.

In conclusion, co-culturing can negatively and positively affect the functionality of hepatocytes and add useful non-hepatic functions, such as anti-inflammatory activity. In this respect, co-cultures of hepatocyte-like cells with MSCs as supportive biocomponent are a promising option.

CONCLUSION

Proliferative human cell sources offer a promising alternative to PHs for BAL application. The hepatoma cell line HepaRG, the modified hepatoma cell line HepG2-GS-CYP3A4, and the differentiated immature cells SHs and HLSCs all demonstrate a substantial level of \textit{in vitro} hepatic functionality when compared to PHs, the golden standard. In addition, HepaRG-BALs, HepG2-GS-CYP3A4-BALs, and MSC-BALs have proven efficacy in well-powered animal studies of ALF. Co-culturing of hepatocytes with supportive cells can improve hepatic functionality and add useful other functions such as anti-inflammatory characteristics. Lastly, only one non-primary human cell-based BAL (the ELAD) has progressed to a clinical trial so far that still awaits its results to be published.
EXPERT OPINION

All of the discussed hepatocyte-like biocomponents show deficiencies in their hepatic functionality compared to PHs. This is probably (at least partly) related to their proliferative capacity and the mutual exclusivity of proliferation and differentiation as found in hepatocytes and other cell sources. A combination of paracrine and autocrine factors and signals from cell-cell and cell-extracellular matrix interactions, e.g. E-cadherin, direct several intracellular signaling pathways, including the Wnt pathway, eventually determine this balance. To circumvent the consequences of the reciprocal relationship between proliferation and differentiation, a number of strategies can be applied.

By overloading the BAL with cells, differentiation can be promoted and proliferation inhibited, as high cell density promotes the establishment of cell-cell contacts, which induces hepatic differentiation. So far, the relation between cell density and differentiation of proliferative biocomponents in BAL devices has not been studied extensively. To our knowledge, our study with two different cBAL111 cell-masses cultured in the AMC-BAL was the only one, indeed showing increased functionality in the high density cBAL111-BAL versus the low density cBAL111-BAL.

Furthermore, hepatic differentiation can be induced by differentiation-inducing growth factors such as HGF, epidermal growth factor, fibroblast growth factor-4, insulin, corticosteroids, and modulators of chromatin structure such as DMSO and butyrate. So far, these compounds have seldom been included in the medium for BAL cultures. Another option is to develop a reversibly immortalized cell line. Kobayashi et al. developed such a line using the hTERT gene under cre-loxP control. Unfortunately, detailed information on the functionality of this cell line is still lacking.

Hepatic differentiation can also be increased by co-culturing hepatocyte-like cells with supporting cells. Promising results have been reported in this respect by coculturing PRHs with MSCs that not only added anti-inflammatory characteristics but also increased albumin secretion. However, coculturing in a BAL system is complex and may even yield negative results as illustrated by the study on the FLC-5/M1/A7-BAL.

Finally, genetic modification may increase hepatic functionality, as illustrated by the beneficial effects of overexpression of OTC, ARG1 and PXR in HepG2 cells, and of HNF4A in OUMS-29 cells. Moreover, BALs based on HepG2 cells overexpressing GS and CYP3A4 have (in contrast to a HepGs-BAL) proven efficacies in an animal model of ALF, underscoring the importance of ammonia elimination and CYP mediated detoxification in BAL therapy.

All these methods to improve hepatic functionality will, however, only be effective when the biocomponent already displays a basic level of hepatic differentiation. With our current knowledge on hepatic differentiation we are still unable to effectively improve biocomponents with low hepatic functionality. Our study on a previously developed, reversibly immortalized cell
Proliferative human cell sources applied as biocomponent in bioartificial livers: a review

line, NKNT-3, on monolayer is exemplary. Reversion of immortalization increased mRNA levels of albumin, transferrin and α-1-antitrypsin 4-20-fold, however, these levels reached maximally 0.1% the level of human liver cells.

Hypothetically, different cell sources may be combined in one BAL to yield a more all-round hepatic functionality. However, from Table 1 it is clear that most biocomponents do not exhibit complementary phenotypes. Instead, most cell sources display synthetic functionality, as determined by albumin expression, and on the other hand share deficiencies in CYP activity, nitrogen and carbohydrate metabolism. This phenomenon probably relates to the liver development in vivo, during which albumin is already expressed early whereas, for instance, CYP genes and the genes encoding the urea cycle enzyme CPS are only expressed late during differentiation.

An important issue regarding BAL therapy is safety. Every cell with unlimited proliferating potential in vitro (particularly cell lines and undifferentiated stem cells) has tumorigenic potential when escaping into the patient’s body during treatment. Therefore, the incorporation of barriers between the cells and the patients is of paramount importance. Genetic modification provides an additional tool to diminish the risk of tumorigenesis by e.g., expression of a suicide gene, such as the herpes simplex virus type 1 thymidine kinase (HSV-tk) gene, that increases the cytotoxicity of the antiviral ganciclovir, enabling efficient elimination of these cells when necessary, as shown in OUMS-29 monolayer cultures.

Taken together, our findings show that several proliferative human cell sources offer a promising alternative to PH cells for BAL application. As yet however, no BAL has proven efficacy in a large randomized controlled clinical trial. Therefore, clinical implementation of a proliferative human cell-based BAL is at least still several years away. Nonetheless, we are confident that the major unmet clinical need to expand our repertoire of therapies for SLF patients will be the driving force to further improve and clinically test the recently developed, promising BALs based on proliferative human biocomponents.
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