Human liver cell lines for the AMC-bioartificial liver
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SUMMARY

Severe liver failure (SLF), comprising acute liver failure (ALF) and acute-on-chronic liver failure, is a syndrome with mortality rates as high as 80%. Clinically, the syndrome presents as a severe impairment of liver function with hepatocellular necrosis, leading to hepatic encephalopathy (HE), systemic inflammation, and multi-organ failure. Despite the progress made in supportive care, liver transplantation is often the only cure, increasing the survival rates to over 80%. However, liver transplantation is limited by the scarcity of donor organs. In the US, about 20% of the patients with SLF who are on the waiting list for liver transplantation die while waiting for a donor liver.

Bioartificial livers (BALs) have been developed to bridge these patients to liver transplantation or liver regeneration. BALs typically comprise a bioreactor that is loaded with a biocomponent with hepatic functionality that is connected to the patient’s circulation. Various BAL systems, based on animal hepatocytes, are efficacious in animal models of ALF. However, due to xenotransplantation-related risks, there is an urgent need for BAL systems relying on human biocomponents.

The main objective of the research described in this thesis was to develop such a human biocomponent for the AMC-BAL, and to test its efficacy in an animal model of acute liver failure (ALF).

Chapter 1 gives a brief overview of SLF and the AMC-BAL. It describes the etiology, pathophysiology, clinical presentation and complications of SLF. Subsequently, it addresses the treatments options for SLF, the need for effective liver replacement therapy, preferably a BAL.

In Chapter 2 we reviewed all literature on proliferative human cell sources that have been applied in BAL systems so far and compared their performance with BALs based on primary hepatocytes. We concluded that, despite the fact that no proliferative human cell source meets the overall hepatic functionality of primary hepatocytes, the hepatoma cell line HepaRG, the hepatoma cell line HepG2-GS-CYP34 (overexpressing hepatic genes glutamine synthetase and cytochrome P450 3A4) (CYP3A4; the most dominant enzyme in hepatic drug metabolism), differentiated immature cells, i.e. ‘small hepatocytes’ and human liver stem cells, display promising in vitro functionality. In addition, BALs based on HepaRG cells, HepG2-GS-CYP3A4 cells, and mesenchymal stem cells have demonstrated efficacy in animal models of ALF. Lastly, various methods increasing the functionality of BALs based on proliferative human cell sources, including genetic modification, the usage of combinatory cell sources, and improvement of culture methods, hold promise, but need further assessment.
The HepG2 cell line is a widely used human hepatoma cell line that exhibits many hepatic functions. Unfortunately, its detoxification functionality (the hepatic transformation of lipophillic endo- and xenobiotics into hydrophilic excretable molecules) is low, whereas a high level of detoxification potential is a prerequisite for a BAL’s biocomponent. In Chapter 3, we evaluated the effects of stable overexpression of the pregnane X receptor (PXR) gene, a master regulator of detoxification, in HepG2, yielding the novel cell line cBAL119. The cBAL119 cell line showed increased transcription of several PXR target genes (49- to 219-fold) and CYP3A4 activity (4-fold) compared to the parental HepG2 cells. Importantly, this increase had not influenced its proliferation potential and several other important hepatic functions, such as its synthetic functionality. Nonetheless, the overall hepatic functionality of the cBAL119 cell line was still too low for BAL application, as e.g. the rate of ammonia elimination was low and inconsistent and the rate of urea production was negligible.

Therefore, we diverted focus to another human hepatoma cell line: the HepaRG cell line. This bipotent liver progenitor cell line differentiates in vitro into two distinct types of cells upon reaching confluence in monolayer cultures: hepatocyte-like cells and cholangiocyte-like cells. Subsequent culturing in the presence of 2% dimethyl sulfoxide (DMSO), probably by modulating the chromatin structure, further increases its detoxification function to a level that is comparable to primary human hepatocytes – so far unparalleled by any other liver cell line. Other hepatic functions that are important for BAL application, such as ammonia elimination, urea production, lactate consumption, and synthetic functionality, had not been investigated yet. In Chapter 4, we characterized HepaRG cells cultured in monolayer, with and without the use of DMSO, and analyzed their potential for BAL application. We demonstrated in this study that HepaRG cells exert a broad array of hepatic functions, e.g. ammonia elimination, urea production, galactose elimination, and synthetic functionality. Unfortunately, the majority of ammonia was not converted to urea, but most likely was fixed into the amino acid glutamine, the latter being a temporary reservoir for ammonia. Interestingly, we also discovered a novel important dual effect of DMSO, while DMSO increased drug metabolism, cell damage was also increased and urea cycle activity was inhibited. Lastly, we demonstrated that ureagenesis of HepaRG cells can be increased by culturing the cells in the presence of carbamoyl glutamate, an analogue of N-acetylglutamate, the physiological allosteric activator of carbamoylphosphate synthetase, the rate-determining enzyme of the urea cycle under normal physiological conditions. We concluded that HepaRG cell line is a promising cell line for BAL application when cultured in the presence of carbamoyl glutamate and possibly when cultures with and without DMSO are combined.
To further study their potential for BAL application we cultured the HepaRG cells in a laboratory scale AMC-BAL, with and without DMSO as described in Chapter 5. HepaRG-AMC-BALs cultured without DMSO eliminated ammonia and lactate, and produced apolipoprotein A-1 (a parameter for synthetic functionality) at rates comparable to freshly isolated hepatocytes. Interestingly, DMSO treatment of HepaRG-AMC-BALs reduced the cell population and the abovementioned functions drastically. Importantly and in contrast to monolayer cultures, CYP3A4 activity was already high in HepaRG-AMC-BALs that were cultured without DMSO, and addition of DMSO did not further increase it. Therefore, solely HepaRG-AMC-BALs cultured without DMSO were tested for efficacy in rats with ALF due to total liver ischemia. HepaRG-AMC-BAL treatment increased survival time of ALF rats with 50% compared to acellular-BAL treatment. Moreover, HepaRG-AMC-BAL treatment decreased the progression of HE, kidney failure, and ammonia accumulation. These results demonstrated that the HepaRG-AMC-BAL is promising for clinical application.

The AMC-BAL provides a superior culture environment compared to monolayer, as culture in the AMC-BAL is three-dimensional, is actively oxygenated, and culture medium is constantly being perfused, as opposed to a static monolayer culture that relies on passive diffusion for oxygenation. We further studied the effects of BAL culture on the HepaRG cells as described in Chapter 6. HepaRG cells in the HepaRG-AMC-BAL are functionally heterogeneous, similar to monolayer cultures, and hepatic functionality of the HepaRG-AMC-BALs increased during 2-3 weeks of culture. The majority of the measured protein-normalized hepatic functions were higher in HepaRG-AMC-BALs compared to monolayer cultures, including ammonia elimination, urea production, and CYP3A4 activity. Interestingly, lactate production in monolayer cultures switched into lactate consumption in the BAL cultures, a hallmark of primary hepatocytes. In addition, protein-normalized cell damage was lower in HepaRG-AMC-BALs, and transcript levels of detoxification-associated genes and hepatic-differentiation-associated regulatory genes reached higher levels than in monolayer cultures. Lastly, metabolism of amino acids, particularly alanine and ornithine more resembled that of primary hepatocytes in HepaRG-AMC-BALs. We concluded therefore that BAL culture of HepaRG cells increases its hepatic functionality, both over time, and compared to HepaRG monolayer cultures.

During treatment of ALF patients, the biocomponent of a BAL is exposed to ALF plasma containing various toxic compounds that may, particularly after prolonged exposure, negatively affect the functionality of a BAL. In Chapter 7, we describe these effects on monolayer cultures of HepaRG cells, either pre-treated with DMSO or not, by exposing them to (undiluted) rat-derived ALF plasma for sixteen hours. As a control, cells were exposed to plasma of healthy rats and to the normal culture medium. Cell leakage only increased in HepaRG cultures without DMSO after exposure to plasma of both healthy and ALF rats. Exposure to rat plasma, even
of healthy origin reduced transcript levels of hepatic genes, urea cycle activity and induced lipid accumulation. Thus, the declined performance of the cells was in general not due to ALF-related components in the plasma, but to the background of the plasma itself, possibly by depletion of compounds normally present in the culture medium. Importantly, the ammonia-eliminating capacity and synthesis of apolipoprotein-A1 proved relatively resistant, underlining the suitability of HepaRG cells for BAL application.

Next, we studied the effects of rat-derived ALF plasma on the HepaRG-AMC-BAL (cultured without DMSO) as described in Chapter 8. We studied the effects of exposure to plasma of rats developing either mild or severe ALF. BALs were tested prior to connection to the rats (control group), after 5 hours of exposure to rats developing mild ALF (mild ALF group), and after 10 hours of exposure to rats developing from mild ALF to death (severe ALF group). Cell damage only marginally increased in both mild and severe ALF groups compared to the control group. Transcript levels of almost all hepatic genes that we studied decreased in both mild and severe ALF groups, particularly regulatory genes and detoxification-associated genes. Interestingly however, hepatic functions mostly remained unchanged in the mild ALF group, or increased in the severe ALF group. All together, the HepaRG-AMC-BAL is relatively resistant to the toxic effects of ALF plasma, with even an (probably compensatory) increase in hepatic functionality. Nonetheless, as we observed a substantial repressive effect of ALF plasma on gene transcription, longer exposure times will ultimately result in a decrease in hepatic functionality.

In Chapter 9, we describe the effect of medium perfusion flow rate on the functionality of the HepaRG-AMC-BAL and on the mass balance of substrates across the bioreactor. To this end we perfused laboratory scale HepaRG-AMC-BALs with culture medium at four different perfusion flow rates (0.3, 1.5, 5, and 10 mL/min) and studied the effects on hepatic functionality (ammonia elimination, urea production, lactate consumption, and CYP3A4 activity) and cell damage parameters. Overall, functionality peaked at a flow rate of 5 mL/min, being 2- to 20-fold higher compared to 0.3 mL/min. The functional differences between the 5 and 10 mL/min groups were small, but at the latter rate, cell damage increased, probably due to increased shear stress on the cells. Therefore, 5 mL/min was the optimal flow rate for the laboratory-scale BAL. When these results are extrapolated to the HepaRG-AMC-BAL for the clinical setting, the optimal flow rate should be approximately 200-250 mL/min.

In addition, we calculated mass balances of ammonia and lactate over the HepaRG-AMC-BAL with the purpose of evaluating their potential in monitoring BAL condition during the treatment of ALF patients. We demonstrated that mass balances between the inflow and outflow medium of the BAL can only be determined at suboptimal, low perfusion rate for the current set-up of the laboratory model of the HepaRG-AMC-BAL.
The HepaRG cell line has been recognized as a promising source for in vitro testing of the metabolism and toxicity of drugs and other compounds. However, the hepatic differentiation of these cells has so far relied on exposure to DMSO, which (as discussed above) has a damaging effect and represses all-round hepatic functionality. In Chapter 10 therefore, drug metabolism of the HepaRG-AMC-BAL cultured without DMSO was evaluated more extensively. The HepaRG-AMC-BALs contained highly polarized viable liver-like tissue with heterogeneous distribution of CYP3A4. We found a substantial metabolism of the tested substrates, ranging from 26% (UDP-glucuronosyltransferase 1A1), 47% (CYP3A4) to 240% (CYP2C9) of primary human hepatocytes. In addition, the HepaRG-AMC-BAL produced bile acids at 43% the rate of primary human hepatocytes and demonstrated hydroxylation, conjugation, and transport of bile salts as well. These results render DMSO addition superfluous for the promotion of drug metabolism. Therefore, AMC-BAL culturing makes the HepaRG cells more suitable for testing metabolism and toxicity of drugs.

CONCLUSIONS AND FUTURE PERSPECTIVES

The major objective of this study was to develop a human biocomponent that, when cultured in the AMC-BAL, is able to effectively replace the failing liver of ALF patients. Therefore, this biocomponent needs to display a high and broad level of hepatic functionality, should be expandable without losing its functionality, and should be safe.

The studies in this thesis show that the HepaRG cell is a very promising biocomponent with respect to functionality. Namely, when cultured in AMC-BAL using a modified cultured protocol with carbamoyl glutamate and without the use of DMSO, it has demonstrated a high and broad level of in vitro hepatic functionality, and more importantly, treatment of ALF rats with this HepaRG-AMC-BAL substantially increased their survival time. Further analysis of the HepaRG-AMC-BAL demonstrated relative resistance to the toxic effects of ALF plasma. In addition, we assessed an optimal medium/plasma flow rate. Therefore, this preclinical work paves the path for clinical studies with the HepaRG-AMC-BAL.

Nonetheless, some important issues need to be addressed before the HepaRG-AMC-BAL can be tested in a clinical trial. First, the results of the rat study should preferably be confirmed by a second study in larger animals using a clinical scale bioreactor. Taking into account that 30-40% of functional liver mass is needed to maintain homeostasis and ALF and ACLF patients generally have some remnant liver functionality, the HepaRG-AMC-BAL should probably contain at least the equivalent of 15-20% of liver mass to be efficacious in ALF and ACLF patients.

In addition, functional stability and safety of the HepaRG-AMC-BAL should be addressed. Functional stability could be studied by prolonged culture of HepaRG cells in the AMC-BAL.
with sequential functionality tests. Safety of the HepaRG-AMC-BAL encompasses toxicological and sterility studies of the HepaRG cells, the bioreactor with and without the HepaRG cells as well as the extracorporeal system as a whole. In addition, as HepaRG cells derive from a hepatocellular carcinoma and are therefore potentially tumorigenic, tumorigenicity studies need to be performed. Even if these indicate a lack of tumorigenicity (as shown in the past), fully controlled filters need to be included in the extracorporeal system to prevent potentially detached cells to enter the body.\textsuperscript{10}

Ideally, BAL therapy should not only bridge ALF and ACLF patients to liver transplantation, but supply sufficient liver support to allow the liver to regenerate, rendering a liver transplantation superfluous. The applied rat model of ALF by inducing complete liver ischemia does not leave any remnant viable liver mass and therefore does not allow liver regeneration. Therefore, a new animal study on the effect of HepaRG-AMC-BAL treatment on liver regeneration is needed. In this respect, animal models based on a subtotal liver ischemia or a subtotal hepatectomy, optionally combined with a toxic ALF model, such as an acetaminophen overdose or a galactosamine / lipopolysaccharide injection, could be useful.

As mentioned in the general introduction of this thesis, the deleterious role of glutamine in the pathogenesis of hyperammonemia-associated HE has been identified only recently.\textsuperscript{11} In short, ammonia is detoxified in the brain by fixation into glutamine, which is transported into the astrocyte’s mitochondrion. Subsequent intra-mitochondrial hydrolysis by glutaminase locally increases ammonia levels that in turn induce oxidative stress, resulting in mitochondrial swelling and dysfunction, leading to alterations in energy metabolism, signalling mechanisms, astrocytic glutamate uptake, and cell swelling. Notably, the main route of ammonia elimination by the HepaRG-AMC-BAL is the less preferred conversion into glutamine and not the permanent conversion into urea. Therefore, the use of glutamine scavengers, such as sodium phenylacetate or sodium benzoate that form conjugates with glutamine that subsequently can be excreted by the kidneys, may reduce glutamine concentrations and thereby add in the treatment of ALF patients with the HepaRG-AMC-BAL.\textsuperscript{12} Alternatively, improving urea cycle activity in the HepaRG-AMC-BAL, \textit{e.g.} by overexpressing genes that encode the rate-limiting enzymes of the urea cycle, may divert the main route of ammonia elimination and thereby decrease glutamine production.

In the search for a suitable biocomponent for the AMC-BAL, we have thus far focussed on hepatocytic functional replacement as a primary goal, as hepatocytes constitute approximately 80\% of the functional liver volume. Nonetheless, non-parenchymal cell types play an important role in liver function as well. The role of Kupffer-cells in ALF and ACLF, the resident macrophages of the liver that scavenge many blood-borne pathogens such as endotoxins, is far from clear. Namely, inhibition of Kupffer-cells can both aggravate or attenuate liver injury, possibly related to different functional subsets that these plastic cells can differentiate into (M1 and M2).\textsuperscript{13} Other hepatic non-parenchymal cells such as endothelial cells and stellate cells have
demonstrated to stabilize and increase hepatocytic functionality in co-cultures, and may offer interesting possibilities for co-culture in a BAL.\textsuperscript{14} Also, mesenchymal stem cells, multi-potent stromal cells that (among others) exert cytokine-mediated immunosuppressing effects, are an interesting candidate for co-culture in this respect.\textsuperscript{15} To be continued.
REFERENCES