Preclinical development of the AMC-HepaRG-Bioartificial Liver

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CHAPTER 8

Summary, conclusions and future perspectives
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SUMMARY

Acute liver failure (ALF) and acute on chronic liver failure (ACLF) are syndromes that are both associated with high mortalities, ranging from approximately 30% up to 96%, depending on disease severity and eligibility for emergency liver transplantation [1, 2]. Liver transplantation is a curative treatment option, but not every patient is eligible and a liver transplant is not available in time for every patient. Even when available, survival after emergency liver transplantation is lower compared to elective transplantation, which is likely due to a worse neurological and inflammatory status of patients in the emergency setting. This means that there is an unmet need for liver support, a need that has been attempted to be met by several means, including high volume plasma exchange therapy, artificial liver support and bioartificial liver (BAL) support. Of these therapies, only high volume plasma exchange has so far proven a benefit on survival in one study: approximately 10% absolute risk reduction of in-hospital mortality in ALF patients in the intention-to-treat analysis [3].

The Academic Medical Center (AMC)-BAL has been under development since the 1990’s. The system was originally based on primary porcine hepatocytes and was under evaluation in a Phase I clinical trial when the group was forced to find an alternative cell source, due to altered xenotransplantation regulation in the early 2000’s. Eventually this alternative was found in the human progenitor cell line HepaRG, and efficacy of the laboratory scale HepaRG-AMC-BAL was proven in a rat model of ALF, due to total liver ischemia [4]. The primary aim for this thesis was to further develop the HepaRG-AMC-BAL towards clinical application.

PART I of this thesis gives an introduction of the AMC-BAL and the selection of its biocomponent for BAL systems.

Chapter 1 starts with a concise introduction on ALF and ACLF, including the definitions, causes and current therapeutic options. Then, the AMC-BAL is introduced with a short history of its development and the current choice of biocomponent: HepaRG.

Chapter 2 is a narrative review on available BAL biocomponents. First, we described the demands posed to cell sources applied in BAL systems when utilized clinically or for in vitro applications, such as drug-toxicity testing. We concluded that a high hepatocyte differentiation grade is important for both applications and that the main differences in demand between in vitro and clinical application are the necessity of a diverse genetic backgrounds in the former and more emphasis on the possibility of economically feasible large-scale production in the latter application. Second, we discussed the available cell sources: primary hepatocytes, liver cell lines and stem-cell-derived hepatocytes. We then systematically reviewed the available literature on stem-cell-derived hepatocyte-like cells and their differentiation grade.
and functional capacity. We found that many studies do not provide sufficient data to fully assess the functional capabilities of hepatocyte-like cells. In the cases where these data were adequately reported, the functional level was several orders of magnitude lower compared to the most functional hepatic cell line available: HepaRG.

Finally, we concluded that for clinical BAL application, HepaRG cells are currently the most suitable cell source, and that there is not yet an all-round superior alternative to primary human hepatocytes for *in vitro* applications.

In **PART II** of the thesis, HepaRG cells and their behavior as a BAL-biocomponent under different culture conditions are further characterized.

In **Chapter 3**, we performed a head-to-head comparison between HepaRG cells and the HepG2 clone C3A that is applied in a BAL that is currently under evaluation in a Phase III clinical trial, but of which little functional data were publicly available. Both cell lines were tested in monolayers and in laboratory scale BALs. HepaRG cells outperformed C3A cells in protein synthesis, ammonia detoxification and xenobiotic metabolism, regardless of culture modality. However, like HepaRG cells, C3A cell performance markedly increased when cultured in BALs. For example, lactate production turned into consumption, and ammonia partly eliminated through the urea cycle by C3A cells only when cultured in BALs. The main conclusion from the comparison was that HepaRG cells are superior to C3A cells for application in a BAL.

**Chapter 4** continues on the observation in Chapter 3 that both HepaRG and C3A cells reach higher hepatocyte differentiation grades when cultured in BALs compared to monolayers. We performed whole-genome microarray analysis on HepaRG cells cultured in monolayers and BALs and found that genes related to mitochondrial biogenesis differed most prominently in their expression, being upregulated in BAL cultures. Increased mitochondrial biogenesis was confirmed by an increase in mitochondrial abundance, mitochondrial membrane potential, protein expression of mitochondrial oxidative phosphorylation complexes as well as transcript levels of mitochondria-encoded genes in BAL- versus monolayer cultures. The three main differences between monolayer and BAL culture are: pericellular oxygenation concentrations, medium perfusion and 2D versus 3D cell configuration. We showed that all three factors contribute to an increase in mitochondrial biogenesis. Finally, we confirmed the stimulatory effect of BAL culture on mitochondrial biogenesis in C3A cells, in addition to HepaRG cells.

**Chapter 5** focusses on the effect of oxygen concentration on HepaRG hepatocyte differentiation in monolayer cultures. We showed that HepaRG cells cultured under hypoxia (5% ambient oxygen) did not acquire a hepatocyte phenotype, but rather represented hepatic
progenitor cells, as indicated by protein expression of progenitor cell marker SOX 9. These effects are likely to be related to oxygen-responsive factors; we confirmed the protein level of one of these factors, Hypoxia-inducible factor 1-alpha (HIF1α), to be higher under hypoxia versus normoxia. HepaRG cells are known to lose their ability to differentiate into hepatocytes around passage 20 from isolation. We showed that HepaRG cells expanded under hypoxia maintained their differentiation potential well beyond 20 passages.

In contrast to hypoxic cultures, cells that were differentiated under hyperoxia (40% ambient oxygen) were morphologically more hepatocyte-like, exhibited increased hepatic functions and nuclear expression of the hepatocyte transcription factor CCAAT/enhancer-binding protein alpha (CEBPα) compared to normoxic cultures. We confirmed these positive effects of hyperoxic culture in C3A cells. Next, we analyzed the transcriptome of HepaRG cells cultured under normoxia or hyperoxia by microarray analysis. Only 240 (of 23,223) probes were differentially expressed between normoxic and hyperoxic HepaRG cultures, including several well-known liver enriched transcription factors. The discrepancy between increased hepatocyte-specific functions and largely unaffected transcriptional activity indicates that the effects of hyperoxic cultures on hepatocyte functions is largely post-transcriptionally.

In Chapter 6, the effects of blood plasma on HepaRG cells is studied. First, we showed that human blood plasma from healthy donors had a dose-dependent toxic effect on HepaRG cells. Within several hours, hepatic gene transcript levels were dramatically downregulated, followed by hepatic functions. Notably, exposure of up to 16 hours did not affect viability. Exposure to different plasma fractions revealed that the effects were mediated by heat-resistant, albumin-bound and (partly) hydrophobic compound(s). Whole-transcriptome microarray analysis of cells exposed to human blood plasma for 30 minutes up to eight hours revealed that the toxic effects of plasma exposure on hepatic gene transcription was within hours and closely correlated with activation of nuclear factor kappa-light-chain-enhancer of activated B cells NF-κB activation, a master regulator of pro-inflammatory cytokine response. HepaRG cells cultured in BALs were less affected by plasma exposure compared to HepaRG monolayers, and in BALs the negative plasma-effect proved to be largely reversible after 16 hours of exposure. The protective effect of BAL culturing was found to be mainly attributable to medium perfusion and 3D configuration. Because of the unavailability of human ALF plasma, we exposed HepaRG cultures to porcine ALF plasma, which elicited a mitotoxic effect, in addition to the detrimental effect of healthy porcine plasma.

In PART III of the thesis, the HepaRG-AMC-BAL was prepared for pre-clinical and clinical testing.
In Chapter 7 we describe the improvement of the logistics and the scaling-up of the HepaRG-AMC-BAL for clinical application. First, the process of large-scale expansion and preservation of the biocomponent needed to be developed. We showed that HepaRG cells can be expanded in monolayer cultures in the Hyperflask™ platform, cryopreserved in large quantities, and seeded directly from cryo-storage into the BAL-cartridge where they underwent two additional population doublings. Second, the BAL cultures were scaled-up from 9mL to 540mL priming volume without loss of functionality, when corrected for total biomass. In addition, we quantified amino-acid depletion during culture to set-up a fed-batch medium supply during BAL culture. Third, the transport conditions of the BAL from production site to the bedside were addressed. Initially, we attempted whole-device cryopreservation, hypothermic and subnormothermic preservation, however, all three strategies resulted in an unacceptable loss of function. To facilitate transport, we therefore successfully developed and tested a clinical-grade transport unit that can maintain temperature, as well as culture medium- and gas perfusion for at least 24 hours. Lastly, tumorigenicity of HepaRG cells was assessed in immunodeficient mice; no animals injected with HepaRG cells developed tumors, while in the positive control group 67% of animals did.

CONCLUSIONS

The objective of this thesis was to better understand and further develop the HepaRG-based AMC-BAL. We gained insight in the roles of 3D configuration, oxygenation, temperature, medium-starvation and plasma exposure on the cells and successfully used this knowledge to develop a clinical size BAL and ditto transport module.

FUTURE PERSPECTIVES

Application of the current generation HepaRG-AMC-BAL

Now that the device is scaled-up, the logical next steps in the application would be a large-animal study and then a Phase I clinical trial. In fact, we have already invested substantial amounts of funds and effort in the development of a paracetamol induced ALF model in pigs to gain data on the efficacy of the HepaRG-AMC-BAL. However, no consistent data could be generated during these studies due to variations in time to occurrence and degree of liver failure, as well as the unexpected lower yield of functional cells inside the bioreactors. Currently, we are looking into the best way to use and represent the data that were collected, although not suitable to judge efficacy of treatment, they may still prove valuable as safety data. The reality of modern market-driven development of innovative medical therapies dictates that in order to gather funds to further develop a product, it must have been applied
in humans first. The HepaRG AMC-BAL has previously proven efficacious in an ALF model in rat [4], and the question arises whether proof of concept in a large animal experimental model is necessary before a Phase I clinical trial is performed.

As discussed in the Introduction, ideally a BAL does not only bridge liver failure patients to transplantation, but to regeneration of the native liver, to avoid a transplantation. A proof-of-principle study for this aim is currently missing for the HepaRG-AMC-BAL, and a survival study should therefore be performed. Several animal models are suitable: subtotal liver resection with/without liver ischemia or toxic models, such as galactosamine + lipopolysaccharide induced liver failure. Ideally, the study set-up should allow treated animals to fully recover from liver injury for a substantial amount of time.

**Further development of the AMC-BAL**

*Hardware*

The AMC-BAL features separate circuits for oxygenation and plasma perfusion, however it lacks a separate drainage system for bile, leading to an accumulation bile components in the patients plasma. The integration of an extra dimension of an artificial biliary tree would involve a high degree of microstructures and cell polarity, a highly complex task that is not likely to be solved in the near future. An achievable alternative is the addition of an albumin dialysis circuit to the BAL set-up to cleanse out (part of) the bile salts and other hydrophobic toxins.

Currently, the design of the AMC-BAL allows treatment using one dosage: one loaded cartridge. However, it has been known for a long time that in humans there is a linear correlation between body surface area and liver size [5], and the degree of remnant native liver function also differs per liver failure patient. It is therefore rational to adapt the amount of biomass to the body surface area and maybe even to the degree of liver damage, although, at this moment, no data are available on how to titrate the latter. Modular BAL cartridges of different sizes and contents that can be combined as needed, would allow for a patient-tailored approach, and could prevent both the use of BALs of insufficient strength, as well as the use of excess amounts of costly biomass.

As described in Chapter 6, the biocomponent is affected by the exposure to patient plasma and, therefore, exposure time should be limited to the functional window of the biocomponent. As this may be variable depending on etiology as well as the individual patient and the moment in disease course, integration of sensor technology into the hardware that can monitor key functions, such as ammonia elimination, markers of cell decay, such as amino-transferases, and/or impedance as a derivative for membrane- or tight-junction integrity [6], will be valuable future additions to the design.
Summary, conclusions and future perspectives

**HepaRG**

As described in Chapter 2, the main detoxification mechanism of ammonia in HepaRG cells is through fixation in the amino acid glutamine through activity of the enzyme Glutamine Synthetase (GS), which has as a disadvantage that the process can be reversed at multiple sites in the human body, including muscles and brain, leading ammonia production. Preferably, ammonia would be converted into urea through urea cycle activity, since this process cannot be reversed in human cells. In the human liver, the majority of hepatocytes exhibits urea cycle activity, a high capacity low-affinity system. Peri-central hepatocytes, however, express, like HepaRG cells, GS, a low capacity, high affinity system that can scavenge residual ammonia not processed peri-portal, located more upstream along the liver sinusoid. The expression of GS and the inhibition of urea cycle rate-limiting enzyme carbamoyl phosphate synthetase 1 (CPS1) are regulated through the WNT/β-catenin pathway [7].

One possible strategy to induce urea cycle enzyme activity in HepaRG cells is by overexpression of CPS1, and possibly other enzymes of the urea cycle. Another way to induce urea cycle activity is through downregulation of β-catenin expression. The difficulty with this approach lies in the broad functional spectrum of the WNT/β-catenin pathway. Many liver tumors have activating mutations in the β-catenin gene, which gives them a growth advantage [8], and, although whole exome sequencing of the (tumor-derived) HepaRG cell line did not reveal mutations in β-catenin gene CTNNB1 (data not shown), it is probable that inactivating β-catenin will have undesired side-effects, such as a decrease in proliferative capacity and, possibly, viability. In fact, in our hands, β-catenin-inhibition appeared to strongly inhibit proliferation in HepaRG cells (data not shown). Possible solutions to this problems include chemical inhibition and transfection with an inducible promotor, although both will require thorough additional safety tests before clinical application.

Once HepaRG cells with increased urea cycle activity have been established, an interesting strategy would be to strive for a zonally designed BAL in a modular bioreactor placed in series to most efficiently eliminate ammonia, as executed along the liver sinusoid. Cells with the current phenotype would be loaded into the distal cartridge, while the proximal cartridge would be loaded with cells that exhibit increased urea cycle activity. Not only ammonia detoxification, but also other functions, such as glucose metabolism and xenobiotic detoxification, are expressed zonally in the liver. Liver zonation enables interaction between specialized cells that is thought to enable the liver to rapidly respond to efficiently maintain homeostasis in, for example, glucose-, nitrogen- and xenobiotic metabolism, while preventing metabolic loops that could appear when opposite functions are expressed simultaneously. Not all of these functions are divided binary into liver zones, nor are they all linked to β-catenin, and in fact, HepaRG cells do not represent peri-central hepatocytes in all respects. However,
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despite the difficulties that are to be anticipated, the road to a zonated BAL can eventually lead to a more efficient device with a broader palette of hepatic functions.

Another strategy to improve HepaRG hepatocyte-specific functions is by overexpression of liver-enriched transcription factors that are low compared to human liver. For example ONECUT1 (4-fold lower in HepaRG versus human liver, data not shown), which forms a transcriptional network with HNF1A and HNF4A, and that is pivotal to hepatocyte development [9]. Finally, the transcription factor Constitutive Androstane Receptor (CAR) is a worthwhile target to explore. Recently, our group produced a HepaRG line that stably overexpressed CAR, which unexpectedly not only induced known CAR-target cytochrome p450 genes, but also induced activity of non-CAR target cytochrome p450 family members and albumin synthesis, as well as a more hepatocyte-like polygonal morphology [10].

**Alternative hepatocyte cell sources**
As discussed in Chapter 2, HepaRG cells are currently the most suitable biocomponent for clinical BAL application. Cell sources with higher differentiation grades are likely to become available in the future, as hepatocyte differentiation protocols keep improving over time. Since publication of the review, several highly interesting papers on novel developments have been published, including the creation of a BAL loaded with Induced pluripotent stem cells (iPSC)-derived hepatocytes [11], and the generation of genomically stable bipotent liver organoids from discarded human liver transplants [12].

Co-culturing of HepaRG cells with other cell types is another method that can potentially improve BALs; co-culturing of primary hepatocytes and non-parenchymal cells has been long known to improve hepatocyte phenotype stability [13]. In C3A cells co-culturing with human umbilical vein derived endothelial cells (HUVECs) resulted in increased protection from acetaminophen toxicity [14], and a combination of iPSC-derived progenitor cells with HUVECs and mesenchymal stem cells resulted in vascularized liver buds that formed viable mini-transplants in mice [15]. Therefore, co-culturing is an interesting option to explore in pursuit of both higher hepatocyte differentiation grade as well as resistance to exogenous factors, such as (ALF-) blood plasma.

**Other novel treatments for liver failure**
This thesis focusses on BAL support as a treatment for liver failure patients, however, others are working on different treatment modalities. Artificial liver support systems envisioned to aid detoxification through albumin dialysis were mentioned previously in this Chapter. Another form of detoxification is aimed at dampening the pro-inflammatory state in liver failure through the use of cytokine scavengers. This method has recently been successfully evaluated in an animal study in combination with albumin dialysis, and a clinical trial is anticipated [16].
Others, yet, are targeting inflammation through macrophage modulation [17], transplantation with mesenchymal stem cells [18], or stimulation of hematopoietic stem cells by injection with granulocyte colony-stimulating factor [19-21]. At this point it is impossible to predict which strategy will be most successful. The treatment may well prove to be most efficacious when multiple approaches are combined.
REFERENCES


