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Improving the next generation of bioartificial liver devices

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Several extracorporeal bioartificial liver (BAL) devices are currently being evaluated as an alternative or adjunct therapy for liver disease. While these hybrid systems show promise, in order to become a clinical reality, BAL devices must clearly demonstrate efficacy in improving patient outcomes. Here, we present aspects of BAL devices that could benefit from fundamental advances in cell and developmental biology. In particular, we examine the development of human hepatocyte cell lines, strategies to stabilize the hepatocyte phenotype in vitro, and emphasize the importance of the cellular microenvironment in bioreactor design. Consideration of these key components of BAL systems will greatly improve next generation devices.

Key words: bioartificial liver / cell therapy / bioreactor / hepatocyte / phenotype

Introduction

The increasing incidence of liver disease coupled with a continual shortage of donor organs for transplantation has spurred the development of many alternative therapies for liver failure. One of the leading approaches, extracorporeal bioartificial liver devices (BAL), has been under development for over 40 years to expedite recovery from acute liver failure or provide a bridge to transplantation. BAL devices typically integrate isolated hepatocytes with membrane-based bioreactors through which a patient's plasma may be perfused. Bioreactor designs aim to maintain cell viability and function without impeding nutrient and metabolite exchange in order to be therapeutically effective.

The first-generation of BAL devices have undergone extensive experimental and clinical evaluation. While recent trials have provided valuable experience in the implementation of BAL support, results have not unequivocally demonstrated efficacy.[1] A number of reviews are available elsewhere that address the history of BAL development,[2,3] therefore, here we will consider the current clinical status of BAL devices and outline issues that may improve the next generation devices such as: cell sourcing, phenotypic stability of hepatocytes, and bioreactor design.

Clinical status of BAL devices

Several BAL devices are currently undergoing clinical testing for the treatment of liver disease (Table 1). In each design, hollow fiber technology is used to separate cellular and perfusion compartments and to provide a basic scaffold for hepatocyte attachment. The most widely tested device, the HepatAssist from Circe Biomedical, integrates cyropreserved porcine hepatocytes on collagen-coated microcarriers in their cartridge as well as a charcoal column in the perfusion circuit for removal of adsorbants. Other variations on the theme include the use of a hepatoma-derived cell line in Vitagen's extracorporeal liver assist device (ELAD)[4] and the multicompartmental interwoven hollow fiber design from Gerlach *et al.*[5]

Ongoing clinical trials have reported that treatments with BAL devices are indeed safe.[6–8] Specific concerns of immune reaction, xenozoonosis, and tumorgenicity have been successfully addressed to gain regulatory approval for introduction to the clinic.[9–11] However, recent results from a large scale, randomized phase II/III trial of the HepatAssist system did not provide unequivocal evidence of efficacy.[1] While treatment groups showed moderate improvement in 30-day survival and time-to-death over control groups, results were confounded by the impact of

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Table 1. Extracorporeal BAL devices in clinical trials

Company	Number of patients	Device	Phase	Comments
Charite Virchow Clinic, Berlin	8	MELS	I/II	Primary porcine hepatocytes, continuous treatment, interwoven hollow fiber design, plasma perfusion, 5
Circe Biomedical	103	HepatAssist	II/III	Cryopreserved porcine, microcarrier-immobilized hepatocytes, intermittent treatment, charcoal column in circuit, plasma perfusion, 1, 8
Excorp Medical	5	BLSS	I/II	Primary porcine hepatocytes, intermittent treatment, whole blood perfusion, 7
Vitagen	25	ELAD	I/II	C3A hepatoma-derived cell line, continuous treatment, plasma perfusion, 4, 6

ELAD: extracorporeal liver assist device, BLSS: bioartifical liver support system, MELS: modular extracorporeal liver system.

transplantation and variations in disease etiology. Thus, as the first randomized, prospective clinical evaluation of a BAL device, the HepatAssist trial is an important milestone that will provide valuable insight for future developments in the field.

As we shall discuss below, improvements in the next generation of BAL devices will be necessary to justify their adoption into the clinic. In addition, careful consideration must be given to the design and implementation of clinical trials. Clearly, the choice of the control arm and clinical endpoints has a large impact on potential results and should be chosen carefully. One alternative to '30-day survival' in patients that are bridged to transplantation may be to treat patient populations that are not eligible for transplant, thus avoiding the major confounding variable, organ availability. Similarly, rather than comparison of BAL treatment to standard medical care, control arms may include treatment with other extracorporeal perfusion systems such as continuous hemodialysis, thereby eliminating negative outcomes related to instrumenting the patient. Finally, the course of liver failure is highly variable and depends on disease etiology. Historically, BAL treatment has been targeted to acute liver failure including acetaminophen toxicity, viral hepatitis, primary graft nonfunction, and acute decompensation on a background of chronic liver disease, to allow for increased enrollment in clinical trials; however, the data now suggest that patients from these subgroups have vastly different rates of spontaneous recovery and may be best analyzed separately. Ultimately, demonstrating efficacy in the relatively small population of acute liver failure patients may lead to treatment of a much larger cohort, those with chronic liver disease.[8,12]

Improving BAL devices

Building a device to replace liver function is a formidable challenge that requires the interdisciplinary efforts of the fields of medicine, biology, and engineering. Considering the current state of BAL technology and ambiguous clinical results, we should re-examine the design and implementation of alternative therapies for liver disease. We outline some of the advances in cell sourcing, stabilizing hepatocyte phenotype, and bioreactor design that may contribute to a new generation of BAL devices that prove effective in the treatment of liver disease.

Cell sources

Primary porcine hepatocytes are the most common cellular component of current BAL devices.[5,13,14] It has been estimated that approximately 15 billion hepatocytes (10% of a normal liver) are required to sustain a patient in liver failure.[15,16] The large numbers of porcine cells required to provide adequate treatment are readily available, but the environmental cues that maintain their liver-specific function *in vitro* are not completely understood.[17] Ideally, primary human hepatocytes could be used in BAL systems, but the high demand for donor organs makes it unlikely that sufficient excess tissue would be available for nontransplant applications. Furthermore, while mature hepatocytes proliferate rapidly *in vivo* during regeneration, primary human hepatocytes divide much less readily *in vitro* even under optimal culture conditions.[18] Research that elucidates the proper culture environment to achieve sustained proliferation of human hepatocytes *in vitro* would revolutionize the

Table 2. Human cells lines for potential use in BAL devices

Cell line	Reported liver-specific function
Tumor-derived	
Hep G2 (C3A)	AFP, Alb, Urea, CYP1A1, 71, 72
HuH6	AFP, Alb, ADH, OCT, 21
JHH-7	AFP, Alb, Fer, Urea, 21
Spontaneous	
HH25, HHY41	AFP, Alb, TF, G6P, CYP1A1, 26, 27
SV40 transformed	
HepZ	Alb, CYP3A4, 73
OUMS-29	Alb, GS, GST, Urea, 21
NKNT-3	Alb, GS, GST, Bil-UGT, 28

Abbreviations: Alb, albumin; AFP, α-fetoprotein; TF, transferrin; GS, glutamine synthetase; G6P, glucose-6-phosphatase; OCT, ornithine carbamoyltransferase; Bil-UGT, bilrubin-UDP-glucuronosyltransferase; CYP, cytochrome P450; GST, glutathione-*S*-transferase; ADH, alcohol dehydrogenase; Fer, ferritin.

field of BAL devices. In the meantime, the development of highly functional, allogenic hepatocytes that can be expanded *in vitro* has been approached by development of tumor-derived cell lines, immortalized cell lines, and attempts to drive stem cells down the hepatic lineage. Table 2 lists some of the human cell lines that may be useful in BAL devices.

Several hepatocyte cell lines have been derived from human hepatic tumors. The C3A subclone of Hep G2 has already been used in clinical trials despite its relatively poor function in key liver functions.[19,20] Other tumor-derived cell lines available for use in BAL systems have only been characterized by their liver-specific gene (and not protein) expression and may not be functionally adequate.[21] The primary safety concern with using tumor-derived cell lines is the transmission of tumorigenic cells to the patient, thus motivating the integration of cell filters in BAL systems as an extra precaution.[22]

Advances in cell biology have enabled the construction of immortal, albeit imperfect, hepatocyte cell lines.[23] One recurring question has been whether one can achieve highly proliferative cells that maintain differentiated functions. While there is some evidence in fetal and regenerative rodent livers that hepatocytes may be uniquely capable of cycling yet expressing liver-specific genes at quiescent levels, this has not yet been observed at the protein level in immortalized cell lines.[24,25] One approach cultured normal human hepatocytes in either a coculture with rat liver epithelial cells or a collagen gel sandwich system and then repeatedly subcultured until the hepatocytes spontaneously immortalized.[26,27] The most common strategy to immortalize cells is the introduction of simian virus 40 tumor antigen (SV40Tag), whose gene product binds to cell cycle regulatory proteins Rb and p53. Two human cell lines, OUMS-29 and NKNT-3, may provide sufficient liver function as shown by improved survival in partially hepatectomized rats receiving cell transplants.[21,28] Recent studies indicate that telomerase activity could play an important role in the immortalization of a primate liver stem cell,[29] but introduction of the protein coding sequence for human telomerase, hTERT, alone may be insufficient to immortalize hepatocytes.[30] Cell cycle regulatory proteins, or cyclins, are another target for immortalization, but transient expression of cyclin D1 and E in cultured hepatocytes has only induced replication in conjunction with other mitogenic factors.[31] Perhaps the best approach for creating a stable, nontumorigenic hepatocyte cell line might combine SV40Tag and hTERT vectors in a reversibly immortalized scheme.[23] Despite the availability of a number of candidate cell lines, functional comparisons are difficult, as most are not characterized at the protein or metabolic level. Furthermore, the biochemical criteria for an 'ideal cell line' remain vague due to the unknown molecular mechanisms that underlie hepatic encephalopathy in liver failure. The 'gold standard' is to reverse hepatic coma with a BAL, a phenomena which can only be observed experimentally.

To address the safety of using transformed cells, investigators have incorporated temperature sensitive constructs, excisable SV40Tag, and suicide genes. Thermolabile SV40T antigen prevents the proliferation of transduced hepatocytes at physiologic temperatures (37–39 °C), but this approach does not disable the immortalizing gene.[32] A more sophisticated system uses Cre/loxP-mediated excision of the SV40 gene after clonal expansion and before treatment application.[28] Though shown effective in animal studies, it is not known whether cells undergoing gene excision protocols could sustain long-term liver-specific function in BAL systems. A final safety measure in immortalized cell lines is the addition of suicide genes, such as HSV thymidine kinase that provide negative selection with gangcyclovir.[33] As immortalization strategies improve and evolve, transformed cells may prove to be a key player in the improvement of BAL devices.

In addition to primary cells and cell lines, stems cells are being considered for use in cellular therapies for liver disease. Potential stem cell sources for use in cell-based therapies are embryonic stem cells,

adult liver progenitors, and transdifferentiated nonhepatic cells.[34,35] While embryonic stem cells could provide a potential source for BAL, differentiation along the hepatocyte lineage *in vitro* has only been reported in murine cells.[36,37] Clearly, availability of stem cells that can proliferate yet retain the ability to differentiate into fully functional hepatocytes would provide an ideal source for BAL devices.

Stabilizing hepatocyte phenotype

Perhaps one of the best ways to ensure efficacy of next generation devices is to improve phenotypic stability of the cellular component. Hepatocytes are attachment-dependent cells that quickly lose their liver-specific function without the proper microenvironmental cues. Current BAL device designs based on hollow fiber technology may adequately maintain cell viability, but do very little to optimize cellular functions. The microstructure of the liver has provided inspiration for culture models that attempt to replace the environmental cues that will maintain liver-specific functions *in vitro*. Modulation of extracellular matrix components, media composition, and cell–cell interactions (both homotypic and heterotypic) used in hepatocyte culture models could greatly benefit BAL designs.

Variations in composition and topology of extracellular matrix (ECM) have been shown to affect hepatocyte stability.[38–40] One method that varies matrix topology termed 'sandwich culture' was designed to mimic the microenvironment of the adult hepatocyte in which cells are sandwiched by extracellular matrix in the Space of Disse.[41] Cells in this configuration express many liver-specific functions for weeks; however, attempts to scale-up this culture method have met with limited success thus far. Matrigel is a biologically derived, basal lamina-like compound that has induced multicellular spheroidal hepatocyte aggregates, which tend to maintain long-term liver function.[42] Though the explicit role of direct cell–ECM signaling as compared to homotypic cell–cell interactions is not clear in this system.

Some modifications of culture media include the addition of low concentrations of hormones, corticosteroids, cytokines, vitamins, or amino acids and are known to help stabilize hepatocyte morphology, survival, and liver-specific functions. Specifically, serum-free formulations containing EGF, HGF, and nicotinamide have been shown to maintain hepatocyte function and even induce proliferation *in vitro*.[43–46] In addition, gradients of hormones and oxygen can modulate hepatocyte function and may prove useful in BAL design.[47,48] However, implementation of nonphysiologic media components may not be applicable to BAL systems that risk systemic exposure to patients.

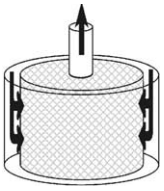
Restoration of cell–cell interactions found *in vivo* has also been reported to stabilize the primary hepatocyte phenotype. Homotypic (hepatocyte/hepatocyte) interactions are promoted in multicellular aggregates created in spheroid cultures or encapsulation schemes.[42,49,50] Aggregates form after a few days in culture on nonadherent substrates, macroporous surfaces, or on Matrigel; however, their utility may be hampered by transport limitations that occur at large aggregate size. This effect has also been noted to increase the function of human immortalized cell lines by three- to fourfold when cultured in encapsulated aggregates, demonstrating the potential for extrapolating the clues gleaned from hepatocyte culture to cell lines.[51]

In addition, the cellular environment of hepatocytes has been modified by the addition of other cell types. Liver-specific functions are stabilized in hepatocytes when cocultured with nonparenchymal cells (heterotypic interaction).[52] Interestingly, both liver-derived and nonliver-derived cell types have been reported to induce phenotypic stability. Liver-derived cell types include rat liver epithelial cells of presumed biliary origin, stellate (fat-storing) cells, sinusoidal liver endothelial cells, Kupffer cells, and the entire 'nonparenchymal' fraction of isolated liver cells.[53] This effect has also been demonstrated to cross species barriers. For example, murine 3T3 fibroblasts, monkey kidney epithelia, canine kidney epithelia, bovine aortic endothelia, and human fibroblasts have been shown, to varying degrees, to stabilize rat hepatocytes.[53] Though the precise molecular mechanisms that underlie the 'coculture' effect are not known, it is likely that this phenomenon represents a highly conserved signaling pathway in development in which contact between the cardiac mesoderm and endoderm foregut initiates liver bud formation.[54–56] Further elucidation of key signals from matrix, soluble factors, and cell–cell interactions that maintain hepatocyte phenotype would greatly contribute to BAL therapies.

Bioreactor design

The design of reactors for application to BAL systems has not suffered from a lack of innovation and creativity. Many of the shortcomings of first-generation devices have been improved in recent designs.

Table 3. BAL device designs

 Hollow fiber	 Flat plate and monolayer
Pros: attachment surface, potential for immunoisolation, well-characterized, cells protected from shear	Pros: uniform cell distribution and microenvironment
Cons: nonuniform cell distribution, transport barrier with membranes or gels	Cons: complex scale-up, potential large dead volume, cells exposed to shear, low surface area to volume ratio
 Perfused beds/scaffolds	 Encapsulation and suspension
Pros: ease of scale-up, promotes three-dimensional architecture, minimal transport barrier	Pros: ease of scale-up, uniform microenvironment
Cons: nonuniform perfusion, clogging, cells exposed to shear forces	Cons: poor cell stability in suspension, transport barrier due to encapsulation, degradation of microcapsules over time, cells exposed to shear forces

Bioreactors can be classified in to four main types: hollow fiber, flat plate or monolayer cultures, perfused beds or scaffolds, and suspension or encapsulation chambers. Each design category has advantages and disadvantages (Table 3), but a successful device should integrate efficient mass transport, scalability, and, of course, maintenance of hepatocyte phenotype.

For the most part, BAL systems have been built around hollow fiber technology that was developed and optimized for use in kidney dialysis. Whether this is the most effective type of bioreactor remains to be seen. Hollow fiber cartridges provide separate compartments for perfusion and cell attachment, but typically do not serve as a semipermeable barrier for transport. Nevertheless, more sophisticated hollow fiber designs that use multicompartmental coaxial or interwoven fibers could perform better than current reactors.[57,58] One experimental large-scale flat plate system uses collagen sandwich culture to stabilize hepatocytes, but may be limited by mass transport and scalability issues.[59] Perfused bed or scaffold reactors designed with glass microcarriers, microchanneled polyurethane, polyester fabric could greatly improve nutrient exchange in BAL systems.[60–62] Some bioreactors have leveraged homotypic stabilization by suspending alginate or hydrogel encapsulated spheroids in perfusion chambers.[63–65]

Independent of the device configuration, BAL systems must provide scalable mass transport. Scalability also infers that a device be capable of housing up to 15 billion hepatocytes needed to adequately support a patient. Many membrane-based reactors can create large diffusion distances and may exclude transport of larger molecules. The inclusion of gels, beads, or other materials intended to stabilize cells provide additional diffusion resistances. BAL devices universally lack an excretory compartment akin to the biliary system that could reduce bilirubin and other metabolic byproducts that accumulate in liver disease. The importance of supplying oxygen to densely packed cells in large bioreactors has been addressed by several investigators.[5,66,67] Overall, mass transport in any bioreactor may face challenging scale-up issues due to increased cell number, dead volume, nonuniform perfusion, and fluid channeling.

Of primary concern in bioreactor design is ensuring that the biological component performs optimally. We have already addressed how various *in vitro* methods are being used maintain hepatocyte function in long-term cultures. In general, the cellular

microenvironment in current devices is inadequate. Some devices are now beginning to incorporate beneficial coculture schemes to boost and stabilize hepatic functions.[57,68] Advances in three-dimensional scaffold fabrication from the tissue engineering field may provide novel approaches to controlling the microenvironmental cues on a large scale.[69,70] Indeed, in the race to show efficacy in the clinic, careful attention to maintaining healthy, high-functioning hepatocytes in BAL devices will be of great benefit.

Summary

The past decade experimental BAL devices have shown promise as an alternative therapy for liver disease. Several clinical trials of first-generation devices are now underway. However, due to recent ambiguous clinical results, it may be necessary to re-evaluate BAL devices, their function, design, and implementation. With the early enthusiasm surrounding the use of cell-based extracorporeal devices waning, the next round of BAL devices must show clear improvement in efficacy and utility.

Some of the key factors that will enable BAL improvement are cell sourcing, maintaining hepatocyte phenotype, and innovative bioreactor design. In light of the scarcity of human liver cells and their limited proliferative potential *in vitro*, new culture conditions that enhance expansion of primary cells, highly functional immortalized cell lines, or efficient differentiation of stem cell sources will be required. Furthermore, careful attention is required to the integration of the hepatocyte microenvironment and bioreactor design that will result in stable, scaleable liver-specific function. As we move forward, it is imperative to integrate our collective past experiences in liver biology, bioreactor design, and clinical treatment of liver disease to develop effective BAL therapies for the future.

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