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Liver Regeneration: the Role of Bioengineering

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1. Introduction

An estimated two million people die of terminal liver disease every year. The World Health Organization calculates that over six hundred and fifty million people worldwide suffer from some form of liver disease, including thirty million Americans. On a worldwide base, approximately one to two million deaths are accounted to liver related diseases annually. Around the globe, China has the world's largest population of Hepatitis B patients (approximately 120 million) with five hundred thousand people dying of liver illnesses every year(1, 2). In the US alone, five hundred thousand critical liver problem episodes are reported every year requiring hospitalization with great burden to the patients and a huge cost to the health care system. In the European Union and United States of America alone, over eighty one thousand and twenty six thousand people died of chronic liver disease in 2006, respectively(1, 3). For these patients, liver transplantation is presently the only proven therapy able to extend survival for end-stage liver disease. It is also the only treatment for severe acute liver failure and to some forms of inborn errors of metabolism. Nevertheless, the waiting list for liver transplantation is long and many patients will not survive long enough to receive an organ due to the dramatic shortage of donors or lack of eligibility(1).

A good example of this is that in 2007 there were almost seventeen thousand candidates on the US waiting list for liver transplantation. From those, only 30% were actually transplanted by the end of the year, with an average waiting time of more than 400 days. In the same year, nearly one thousand and three hundred people died while waiting for a suitable donor, with no real therapeutic alternative available to save their lives. Moreover, for those patients with fulminant hepatic failure, a severe liver disease with 60-90% mortality, depending on the etiology, only 10% received a transplant. Altogether, liver transplantation still has a relatively high mortality of 30-40% at 5-8 years with 65% of the deaths occurring in the first 6 months. Patients who have undergone transplantation have to also use lifelong immunosuppressive therapy, with sometimes severe side effects(4).

There are innumerous etiologies of end-stage chronic liver disease that lead to transplantation and approximately 80% of the candidates in the liver transplantation waiting list have a primary diagnosis of liver cirrhosis. Fortunately, some of the causes of these diseases are currently preventable. An excellent example is the successful vaccination programs in many countries around the world against Hepatitis B virus, which have

considerably reduced the incidence of chronic carriers and viral induced cirrhosis(5). However, close to 20% of the livers transplanted in the USA and 30% in Europe have a preventable underlying cause, alcoholic liver disease. Furthermore, approximately 45% of deaths due to liver cirrhosis in the USA are associated with alcohol abuse(1, 3, 4). Patients with pathologies like hepatic cancer, congenital malformations and metabolic diseases, and acute hepatic necrosis make up the remaining percentage of the list.

The success of liver transplantation has resulted in a progressively increasing demand for such treatment. Nevertheless and as mentioned above, the availability of donor organs has remained stable, resulting in the number of potential recipients far exceeding organ supply. Due to this, several strategies have been explored in the past decade or so with the aim to increase access to liver transplantation. These consist of obtaining organs from non-heart-beating and live donors; and/or using split liver technique and livers from expanded donor criteria. In addition, the introduction of the Model for End-Stage Liver Disease (MELD) score system implemented on February 27 of 2002 in the United States tremendously helped Organ Procurement Organizations to prioritize patients waiting for a liver transplant. The MELD score is a numerical scale used for adult liver transplant candidates that ranges between 6 (less ill) and 40 (gravely ill). The number is calculated using the most recent laboratory tests for bilirubin, INR and creatinine(6) and the individual score determines how urgently a patient needs a liver transplant within the next 3 months.

The implementation of more adequate and efficient allocation systems, development of better immunosupressive regimens, and the increase of living donors have all helped to raise overall patient survival and graft survival in the past decade in the United States. The number of transplanted livers also increased to an all time high in 2006, with a marked decrease on the waiting time for liver transplantation after MELD score system implementation, especially for the sickest patients.

The best example of the impact of these measures is the increase of 6% (86% in 2007) and 16% (87% in 2007) of the unadjusted 1-year graft survival for deceased donor and living donor liver recipients between 1998 and 2007, respectively. This explains also the improvement of 3% (89% in 2007) and 11% (91% in 2007) of the unadjusted 1-year patient survival for deceased donor and living donor liver recipients for the same period, respectively(7). However, these numbers decrease significantly when we consider 5-year patient survival, showing how much remains to be done. In 2007 it was 74% and 79% for deceased donor and living donor liver recipients, respectively. These numbers decrease even further for the 10-year patient survival, where in 2007 there were 61% and 71% patient survival for deceased donor and living donor liver recipients, respectively. One important note is that patient survival was higher than graft survival ~5%, due to the opportunity for repeated liver transplantation in the event of graft failure(8).

These numbers highlight the need for innovative therapies that can increase patient survival, as well as lower costs to the health care systems. Naturally acquired tolerance research and its experimental clinical induction is a good example of this. The identification of molecular signatures in naturally tolerant patients to whom immunosuppression could be stopped, and of tolerance induction, through lymphocyte depletion or T lymphocyte co-stimulation blockade, are some of the most advanced approaches to decrease the complications of immunosuppression(9).

Additionally, artificial liver devices which can efficiently remove accumulated lethal toxins from blood or plasma by using membrane filtration and/or adsorbents have been developed as support devices. Liver Dialysis Device, Molecular Adsorbent Recirculating

System (MARS) and Prometheus are the most widely used artificial support systems. These devices have been widely used in clinical trials across Europe and Asia, and have showed some benefits to the patients, but unfortunately have little or no significant improvement in patient survival.

2. Bioartificial Liver Devices (BALs)

Extracorporeal liver support devices have been developed in the past few decades to support the failing liver resulting from different complications. These devices were created initially for the management of patients waiting for a suitable donor for orthotopic liver transplantation. Recent advances in the design of these devices have made it possible to utilize them for recovering the liver from an acute injury. Thus, these devices can either bridge the patients to liver transplantation or can fully avoid the need for it (10).

Although artificial liver devices have been able to provide temporary support to the patients with acute liver failure by detoxifying the blood or plasma, they have major limitations in replacing synthetic and metabolic functions of liver (11). Thus, attempts have been made to develop bioartificial liver systems, which can provide both metabolic and synthetic hepatic functions along with detoxification. BALs generally utilize primary hepatocytes or hepatoma cell lines as a biological component and a hollow fiber or porous matrix membranes on which the functional hepatocytes are coated (11, 12). Hepatocytes from various sources have been investigated for use in BALs. Primary human hepatocytes have been widely studied as an ideal cell source due to their biocompatibility but they are scarcely available and their proliferative capacity *in vitro* is limited (12, 13). Animal cell sources such as porcine primary hepatocytes are being investigated due to ease of availability and their ability to maintain metabolic functions similar to human hepatocytes. However, concerns regarding immunological reaction to the animal proteins and transmission of disease exist (14). Nonetheless, porcine hepatocytes remain a popular choice as a hepatocyte source in various BAL systems.

A bioreactor is a critical component of BALs and thus has a major impact on the efficacy of these systems. The bioreactor should be capable of providing a suitable environment for hepatic cells to survive and remain functional along with an adequate interface between blood and hepatocytes for mass transport (15). The bioreactors should also be flexible enough for scale up and customization according to the patient's needs. Currently available bioreactor systems need structural optimization and modifications even though there have been recent advances in this technology. It should be highlighted that no bioreactor system is currently approved for patient use, although some have been used in clinical trials (16). Table 1 lists all BAL devices currently under clinical investigation.

So far over 200 patients have been treated with HepatAssist and over 40 patients treated with Extracorporeal Liver Assist Device (ELAD), making them the most common BALs used in clinical trials so far (22, 23). In all these cases, most patients were bridged to liver transplantation while some patients fully recovered thus avoiding the need of transplantation. ELAD is the only BAL system which utilizes a human hepatocyte cell line; most of the other BAL systems use porcine hepatocytes as a cell source. ELAD uses the immortalized C3A cell line derived from human hepatoma cell line HepG2 (24). The cells are located in the extracapillary space of hollow fiber cartridges (200 gram total cells in four cartridges). The membrane is impermeable to immunoglobulins, blood cells and C3A cells. The blood flows through the lumen of cartridges as the ultrafiltrated plasma from the

membrane comes in direct contact with hepatocytes (11). HepatAssist incorporates approximately 5-7 billion cryopreserved porcine hepatocytes attached to microcarriers and loaded onto a hollow fiber. The separated plasma passes through a charcoal column and oxygenator prior to entering the hollow fibers in the bioreactor. An upgraded version of HepatAssist known as Hepamate contains $14x10^9$ porcine hepatocytes. The membrane pores are 0.15µm in size which prevents a physical contact between human cells and porcine hepatocytes (10, 23). Most of the BALs listed above are undergoing clinical trials in the USA, Europe and Asia. The goal of these clinical trials is to assess the safety and efficacy of these devices in treatment of various terminal liver diseases. Currently, none of the BALs have been approved by the FDA for clinical use.

Device	Reference
Extracorporeal Liver Assist Device (ELAD)	(17)
HepatAssist	(18)
Bioartificial Liver Support System (BLSS)	(19)
The Academic Medical Center – Bioartificial Liver (AMC-BAL)	(20)
Modular Extracorporeal Liver Support device (MELS)	(21)

Table 1. Summary of developed and published bioartificial devices.

Recent developments in artificial and bioartificial devices have shown a potential for use of these devices in the management of patients with acute liver failure. However, considerable technical challenges and regulatory issues remain to be addressed in order to efficiently utilize these devices in the clinic. Artificial liver devices have demonstrated the ease of use and cost effectiveness along with showing improvement in biochemical parameters and clinical symptoms by detoxifying the blood or plasma, but it has a major limitation of not replacing critical metabolic and synthetic functions of liver. BALs developed over the past decade have been designed to provide these functions of the liver along with detoxification. BALs hold a promising future as they have shown potential by efficiently treating several patients across different clinical trials. Many challenges exist in BAL technology including the debate on ideal cell source, the requirement of a large number of cells, maintainance of the functional hepatocytes for a longer period of time in a bioreactor, complexity of the design and high cost. The aforementioned challenges have delayed the entry of BAL

systems in the clinic. Nonetheless, there are plenty of optimized designs of liver support devices that are undergoing development and clinical trials. This is an unmistakable sign of optimism in this area of critical care management.

3. Cell therapies

Hepatocyte transplantation is certainly in the vanguard of new therapeutic strategies. The first successful hepatocyte transplantation was performed in June 1992 to a French Canadian woman with familial hypercholesterolemia. After *ex vivo* transduction with a retrovirus encoding for the human LDL receptor, the patient's hepatocytes were infused through the inferior mesenteric vein into the liver. LDL and HDL levels improved throughout the next 18 months and transgene expression was detected in a liver biopsy(25). Following this first accomplishment, other patients followed through. However, not all the patients treated had a clear benefit from the procedure(26).

Since then,several other metabolic diseases have been treated with hepatocyte transplantation with various degrees of success(27-31). It has also been used as a support treatment to acute(32-34) and chronic liver diseases(33-36) in bridging severely ill patients to orthotopic liver transplantation (OLT). Low efficacy and lack of long-term therapeutic effect have been common in all these procedures. These failures could be explained by the relatively small number of hepatocytes that engraft in the recipient liver due to quality, quantity and possibly immunosuppression protocols(37). However, transplantation of a number of hepatocytes corresponding to 1-5% of the total liver mass has been able to show a positive impact in transplanted patients, even if for a limited period of time(37).

Due to the shortage of available human hepatocytes for transplantation, other cell sources have been used. Specifically, bone marrow derived mesenchymal stem cells(38), hematopoietic stem cells(39, 40) and fetal liver progenitor or stem cells (41) have shown to improve, to a certain extent, the condition of cirrhotic patients. The latter cell type holds an enormous potential for cell or regenerative medicine therapies due to their high expansion capabilities and differentiation into hepatocytes and biliary epithelium(42).

Cell Type	Disease	References
Hepatocytes	Familial Hypercholesterolaemia	(25, 26)
Hepatocytes	Crigler-Najjar syndrome Type I	(27)
Hepatocytes	Severe Ornithine Transcarbamylase Deficiency	(28)
Hepatocytes	Crigler-Najjar Syndrome Type 1	(29)
Hepatocytes	Glycogen Storage Disease Type 1a	(30)
Hepatocytes	Peroxisomal Biogenesis Disease	(31)
Hepatocytes	Acute Liver Disease	(32-34)
Hepatocytes	Chronic Liver Disease	(33-36)
BM-Mesenchymal SC	Chronic Liver Disease	(37)
Hematopoietic SC	Chronic Liver Disease	(39, 40)
Fetal Liver Progenitor/ Stem Cells	Chronic Liver Disease	(41)

Table 2. List of cell therapy procedures that were performed in clinical trials

Recent data suggests that human embrionic (hES) and induced pluripotent (iPS) stem cells hold great promise to regenerative applications in every medical field. Specifically for the

liver, several studies have established the required pathways to differentiate a hES or iPS into a hepatic fate by using defined soluble growth factor signals that mimic embryonic development(43, 44). These cells, once transplanted into rodent livers were able to engraft and express several normal hepatic functions(45, 46). Still, more extensive characterization, as well as further safety evaluation, are needed to determine whether these cells can fully function as primary adult hepatocytes.

4. Tissue and organ bioengineering

Tissue engineering is one of the most promising fields in regenerative medicine. As described in 1993 by Robert Langer and Joseph Vacanti, it is the conjugation of biomaterials (synthetic or naturally derived) with cells, in order to generate tissue constructs that can be implanted into patients to substitute a lost function, and maintain or gain new functions(47). The current paradigm is suitable for the engineering of thin constructs like the bladder, skin or blood vessels. Although, in the specific case of the liver, the 3D architecture and dense cellular mass requires novel tissue engineering approaches and the development of vascularized biomaterials, in order to support thick tissue masses and be readily transplantable. Additionally to the vascular support for large tissue mass, hepatocyte function maintenance represents the ultimate aim in any organ engineering or regenerative medicine strategy for liver disease.

Hepatocytes are known to be attachment-dependent cells and lose rather quickly their specific functions without optimal media, ECM composition, and cell-cell adhesion. Also, function and differentiation of liver cells are influenced by the 3D organ architecture(48).

In the last two decades innumerous strategies for the culture of adult hepatocytes in combination with several types of 3D, highly porous polymeric matrices have been attempted(49-53). Nevertheless, lack of vasculature, restriction in cell growth and function are common due to the limitations in nutrient and oxygen diffusion. Finally, some of these problems have been partially resolved with the development of bioreactors that provide continuous perfusion of culture media and gases allowing a 3D culture configuration and hepatocyte function maintenance(54-56).

The tissue engineering concept has several advantages over the injection of cell suspensions into solid organs. The matrices provide sufficient volume for the transplantation of an adequate cell mass up to whole-organ equivalents. Transplantation efficiency could readily be improved by optimizing the microarchitecture and composition of the matrices as well as by attaching growth factors and extracellular matrix molecules to the polymeric scaffold, helping to recreate the hepatic microenvironment(48). The use of naturally derived matrices has also proved to be very helpful in hepatocyte culture(51). These matrices, besides preserving some of the microarchitecture features of the tissues that they are derived from, also retain bioactive signals (e.g., cell-adhesion peptides and matrix bound growth factors) required for the retention of tissue-specific gene expression(57, 58). Additionally, cell transplantation into polymeric matrices is, in contrast to cell injection into tissues and organs, a reversible procedure since the cell-matrix-constructs may be removed if necessary.

Finally, heterotopic hepatocyte transplantation in matrices has already been demonstrated in long-term studies(59, 60), even so initial engraftment rates are suboptimal. One of the reasons for this is the absolute requirement of the transplanted hepatocytes for hepatotrophic factors that the liver constantly receives through its portal circulation(61). Thus, the development of a tissue engineered liver construct capable of being orthotopically transplanted is essential.

Apart from cellular therapies, other early developments of experimental approaches are not showing results that will indicate clinical translation in the next few years. However, two experimental approaches are worth mentioning. They already display a higher functional level and may have the potential for succesful clinical translation. The first experimental approach is the "cell sheet" technology developed by Okano *et al*. in Japan(62). Its simple configuration and fabrication allows for the stacking of up to four hepatocyte cell sheets that can readily engraft and provide a specific metabolic relief to the recipient(63). This technology has already been applied successfully to one patient with heart failure. Other technology that shows great promise is tissue and organ decellularization. Our lab and others have been able to generate several decellularized scaffolds for tissue engineering applications like tissue engineering of urethra(64), heart valves(65), blood vessel(66). More recently, Ott *et al*. reported a novel method of perfusion decellularization that is able to generate whole organ scaffolds. The use of this method allowed the decellularization of a whole heart that was later repopulated with neonatal rat cardiomyocytes. This bioengineered heart was able to contract up to 2% of the normal contractile function(67). This approach may have a tremendous potential for the field of organ bioengineering.

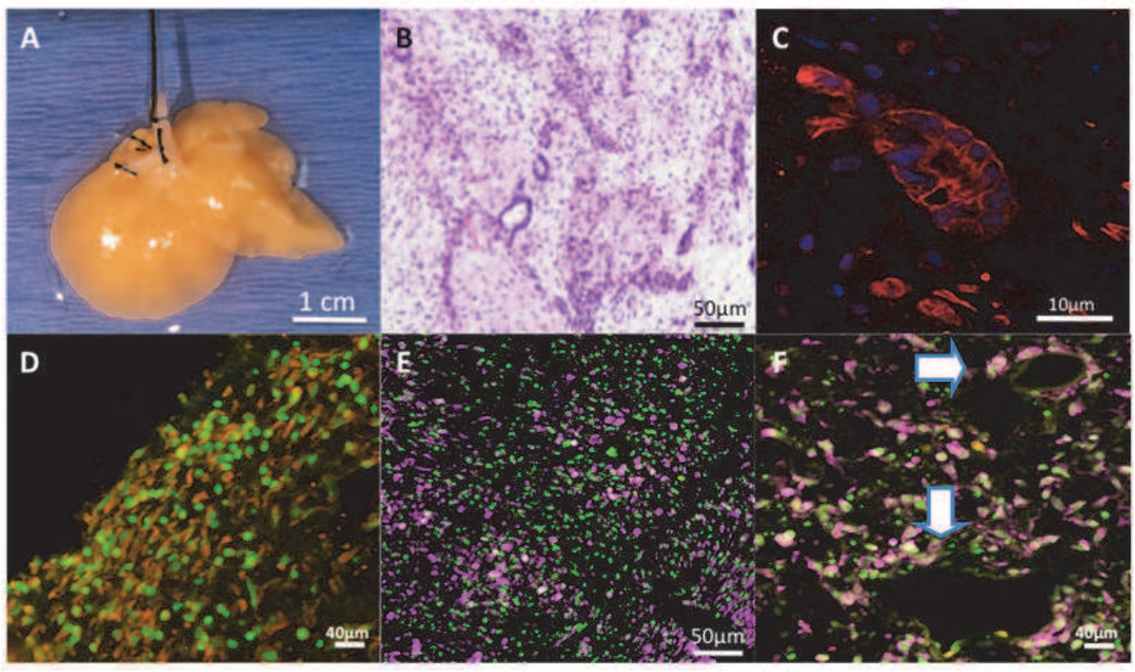

Fig. 1. Human bioengineered livers are highly cellular and display some of the functions observed in native hepatic tissue. (A) Macroscopic appearance of a seeded liver bioscaffold 7 days after seeding with primary human fetal liver and endothelial cells. (B) H&E showing broad recellularization of the bioscaffold with the formation of biliary ductal structures. (C) Immunofluorescence staining for cytokeratin 19 (red) showing a biliary duct formed within the bioscaffold with a visible lumen. (D) Immunofluorescence staining of cytochrome P450 3A (orange) and (E) albumin (purple) showing groups of hepatocytic lineage cells expressing these more mature hepatic markers. (F) Immunofluorescence staining of eNOS (purple) showing hUVECs coating and spreading from vascular structures (arrows). All nuclei stained with YO-PRO1 (green) or DAPI (blue).

We have also developed a similar perfusion decellularization method for the liver, reported for the first time in June 2005 (68), which culminated with two papers published recently(69, 70). We applied this technique to liver, pancreas, intestine and kidney generating decellularized organ scaffolds for organ bioengineering(69, 71). These bioscaffolds preserve their tissue microarchitecture and an intact vascular network that can be readily used as a route for recellularization by perfusion of culture medium with different cell populations. In the particular case of the bioengineered liver, the generated hepatic tissue is clearly visible by the naked eye after 7 days in the bioreactor (Fig. 1A). This tissue is highly cellular (Fig. 1B) and displays biliary duct structures positive for cytokeratin 19 (Fig. 1C), as well as clusters of hepatocytes expressing cytochrome P450 3A and albumin (Fig. 1D, E, respectively). The seeded human endothelial umbilical vein cells (hUVECs) are also observed coating and spreading from vascular structures and express endothelial cell nitric oxyde synthase (eNOS) (Fig. 1F).

In a similar approach, Uygun *et al*. decellularized rat livers and repopulated them with rat primary hepatocytes, showing promising hepatic function and the ability of heterotopically transplanting these bioengineered livers into animals for up to eight hours(72). Nevertheless, we were able to take this a step further in our lab by using human primary fetal liver progenitor/stem and endothelial cells to bioengineer a vascularized human liver. Additionally to the markers showed above, these bioengineered livers displayed some of the functions of a native human liver (albumin and urea secretion, CYP450 enzyme expression, etc) and also exhibiting an endothelialized vascular network that prevented platelet adhesion and aggregation, critical for blood vessel patency after transplantation(71). Hence, this technology has the potential to translate in the future into the bioengineering of human size livers, which may offer readily available organs for drug discovery applications and for transplantation, overcoming organ shortage.

5. Conclusion

OLT is the only cure for end-stage liver disease and inborn errors of metabolism. Due to lack of appropriate organ donors the new experimental procedures presented here have the potential to replace liver transplantation and become the standard of care. Regenerative medicine has indeed the potential to simplify and reduce the morbidity or impact of the "life changing" procedure of OLT.

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This book provides an example of the successful and rapid expansion of bioengineering within the world of the science. It includes a core of studies on bioengineering technology applications so important that their progress is expected to improve both human health and ecosystem. These studies provide an important update on technology and achievements in molecular and cellular engineering as well as in the relatively new field of environmental bioengineering. The book will hopefully attract the interest of not only the bioengineers, researchers or professionals, but also of everyone who appreciates life and environmental sciences.

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