Hepatic tissue engineering: from transplantation to customized cell-based liver directed therapies from the laboratory

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Abstract

Today, liver transplantation is still the only curative treatment for liver failure due to end-stage liver diseases. Donor organ shortage, high cost and the need of immunosuppressive medications are still the major limitations in the field of liver transplantation. Thus, alternative innovative cell-based liver directed therapies, for example, liver tissue engineering, are under investigation with the aim that in future an artificial liver tissue could be created and be used for the replacement of the liver function in patients. Using cells instead of organs in this setting should permit (*i*) expansion of cells in an *in vitro* phase, (*ii*) genetic or immunological manipulation of cells for transplantation, (*iii*) tissue typing and cryopreservation in a cell bank and (*iv*) the *ex vivo* genetic modification of patient's own cells prior to re-implantation. Function and differentiation of liver cells are influenced by the three-dimensional organ architecture. The use of polymeric matrices permits the three-dimensional formation of a neo tissue and specific stimulation by adequate modification of the matrix surface, which might be essential for appropriate differentiation of transplanted cells. In addition, culturing hepatocytes on three-dimensional matrices permits culture in a flow bioreactor system with increased function and survival of the cultured cells. Based on bioreactor technology, bioartificial liver devices (BAL) are developed for extracorporeal liver support. Although BALs improved clinical and metabolic conditions, increased patient survival rates have not been proven yet. For intracorporeal liver replacement, a concept that combines tissue engineering using three-dimensional, highly porous matrices with cell transplantation could be useful. In such a concept, whole liver mass transplantation, long-term engraftment and function as well as correction of a metabolic defect in animal models could be achieved with a principally reversible procedure. Future studies have to investigate which environmental conditions and transplantation system would be most suitable for the development of artificial functional liver tissue including blood supply for a potential use in a clinical setting.

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Introduction

Today, liver transplantation is an established and successful procedure that represents the only causal and curative therapy for many liver diseases that lead to liver cirrhosis and consecutive liver failure in end-stage disease [1]. Despite its therapeutic potential it remains an unspecific approach that is limited by donor organ shortage [2] and the need for a life-long immunosuppressive therapy with its specific risks [3]. According to data from the UNOS Database 6134 livers have been transplanted in 2006, but in March 2007 16,995 candidates where on the waiting list [4]. This situation makes the search for alternatives to whole organ transplantation an important topic in current transplantation research.

Extracorporeal systems seem to be suitable for acute intervention in cases of acute liver failure (ALF) or intoxication, and may serve as a bridge to liver transplantation or organ recovery [5]. However, the long-term application of this concept is problematic due to technical limitations, high costs and the necessity of an intensive care unit setting.

Many liver diseases with primary intact liver function and organ architecture require only the correction or replacement of a small sector of the complex liver function that may in future be accomplished by gene therapy [6]. The transplantation of a hepatocyte mass equivalent to 10% of the patients' liver would be sufficient to normalize the metabolic situation in many enzyme deficiencies [7]. For this purpose intracorporeal systems based on the transplantation of isolated liver cells are desirable. Cell transplantation has some advantages over organ transplantation: one donor organ could be used for many candidates. Because this is a much lesser invasive technique it is associated with a lower mortality and morbidity. Cell transplantation is also thought to be less immunogenic because transplanted allogenic cells could be immunomodulated prior to implantation or even autologous cells might be utilized following *in vitro* modification. Cell transplantation might be an option for patients with metabolic diseases because the complete organ does not need to be replaced and the deficient metabolic function could be replaced or at least supported by only a small portion of hepatocytes.

Development of cell isolation and primary culture for hepatocytes

Cell-based therapies for liver failure ideally require proliferative cells with the capability to differentiate into different types of liver cells. Over the past 50 years a variety of methods have been developed for the isolation of liver cells. The first isolation techniques resulted in a high percentage of damaged cells because mechanical force was used. Anderson *et al.* used a Ca^{2+}-free solution for perfusion of the liver under pressure [8]. Many of the cells isolated this way were damaged. When in 1967 Howard, Christensen, Gibbs and Pesch [9] introduced the enzymatic method of cell isolation, a milestone in hepatocyte isolation was achieved. Berry and Friend [10] modified this method in 1969 and perfused the rat liver via the portal vein for the first time. They were able to convert almost 50% of the rat's liver into viable, intact hepatocytes. Seglen *et al.* improved this method and used a two-step perfusion [11]. Physiological liver perfusion leads to a high yield of intact liver cells, and most efforts in liver cell isolation since Berry and Friend have been dealing with the optimization of temperature, collagenase and Ca^{2+} concentration [12]. The intra- or extracorporeal physiological liver perfusion still is the state-of-the-art technique used for liver cell isolation. Non-enzymatic methods are also considered as a feasible technique for the isolation of adult hepatocytes [13–15]. Other cell types that can be used for hepatic tissue engineering include hematopoietic stem cells, oval progenitor cells, adult hepatocytes and hepatoblastoma-derived cells. Initial data indicate that mesenchymal stromal cells (MSCs) might also generate hepatic progenitor cells *in vitro* under the appropriate culture conditions [16–18]. The factors that are needed for differentiation include growth factors, cues from other cells and extracellular matrix molecules (ECMs). The special conditions in which MSCs can be transdifferentiated into hepatocytes and other cell types are currently investigated [19–21].

Three-dimensional culture using matrices

Hepatocytes are attachment-dependent cells and lose their liver-specific function without optimal media- and ECM composition and cell-cell contacts. In order to develop potent culture systems for hepatocytes, hepatotrophic stimulation of the cells *in vitro* is necessary [22]. Several stimulatory mechanisms are evaluated in hepatocyte cultures:
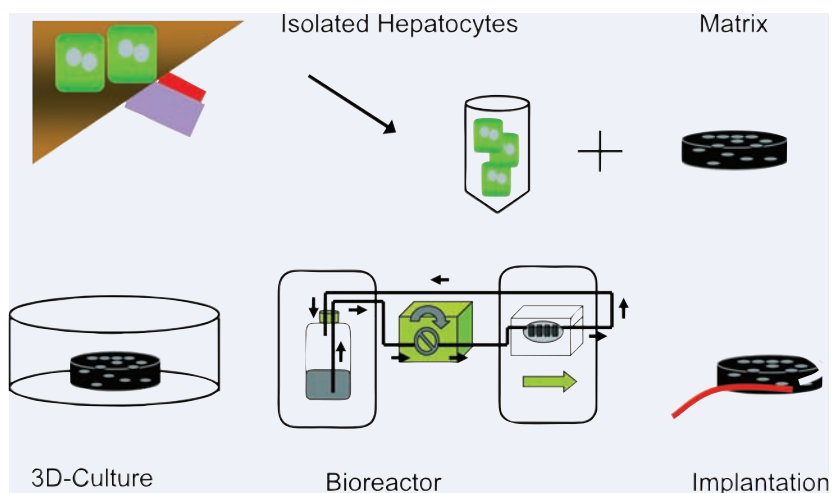
Fig. 1 Scheme of the tissue engineering approach for the liver. Isolated liver cells are seeded on three-dimensional matrices in order to stimulate cell proliferation and hepatocyte-specific differentiation. The cell-seeded matrices can be used for successful three-dimensional culture, culture in a bioreactor system and for transplantation.

(*i*) Coating of culture dishes with isolated ECMs [23, 24], (*ii*) the addition of growth hormones and cytokines to the culture media [25, 26] or (*iii*) co-culture with other cell types [27–30]. In particular the culture configuration has been shown to have a major impact on cellular differentiation: cultures in 'sandwich' configuration could achieve a significant elongation of the culture period, as well as an increase of specific function and cell growth [24]. A variety of novel culture systems for hepatocytes, including hydrogel microspheres, hollow fibres and macroporous polymer scaffolds were developed and shown to promote specific functions, such as albumin secretion or detoxification capacity [31, 32]. Furthermore, initial data suggest a strong positive influence caused by flow in a bioreactor system for hepatocyte culture: Hepatocytes cultured under flow conditions show new tissue formation and high albumin production [33]. This approach seems to be attractive, because it may permit the creation of a functional BAL tissue for transplantation (Fig. 1).

Development of bioreactor systems for liver cells

Currently, adult hepatocytes used for tissue engineering include those derived from immortalized human hepatoblastoma cell lines such as HepG2/C3A. The main advantage is the easy cultiva-tion of large quantities of those cells. When discussing the clinical usage of tumour cell lines the long-term safety is still an important issue, which should be addressed in further studies before clinical use.

For most available BAL systems, porcine hepatocytes are being used. The advantage of these cells is their cheap and easy availability. As long as the porcine hepatocytes are kept outside the patients' circulation there is no danger of immunological reactions. For cell transplantation the possible usage of genetically altered porcine hepatocytes that lack α-galactose is currently discussed [34]. For cell culture different types of bioreactors exist. For BAL devices mostly hollow fibre bioreactors loaded with porcine hepatocytes are used, but there also exist monolayer bioreactors, perfused scaffolds and cell suspensions. The hollow fibre technology, which was developed for kidney dialysis, is an easy technology for BAL. Cells are protected from shear stress and a high number of cells can be cultivated in a small volume because of a big attachment surface [35].

Monolayer cultures using the 'sandwich culture' show good stability of hepatocytes, but the cells are exposed to shear stress when the culture is perfused [36]. These cultures also have a low surface to volume ratio. Perfused scaffolds such as PLGA [37] also show the problem of shear forces; the advantages include ease of scale-up and the positive effects of three-dimensional architecture of cell culture. Suspension cultures show poor cell stability but ease of scale-up and very good transfer between plasma and immobilized hepatocytes [38, 39].

Table 1 Clinical trials of BAL devices

Device	Cell Source	Study Type	Patients Treated	Comments
ELAD	C3A-cells (hepatoma-derived)	Phase I/II	52 [45, 46]	
HepatAssist	Porcine	Phase II/III	171 (86 patients. In control group, 85 received BAL treatment) [47]	Survival advantage in FHF
MELS	Human	Phase I	20 [49, 50]	Patients bridged to transplantation; system based on BELS (which was based on porcine hepatocytes)
AMC-BAL	Porcine	Phase I	8 with acute HF [53]	7 patients bridged to transplantation, 1 recovered
BLSS	Porcine	Phase I/II	4 [82]	Decreased bilirubin, lactate and ammonia levels

BELS, bioartificial extracorporeal liver support.

First clinical application of bioreactors with liver cells

First attempts in artificial liver support were based on charcoal hemoperfusion [40]. Newer systems such as MARS (molecular adsorbents recirculating system, Gambro, Sweden; developed by Stange *et al.* [41]) and FPAD (Fractionated Plasma Separation, Adsorption and Dialysis system, Prometheus, Fresenius Medical Care, Bad Homburg, Germany) eliminate protein-bound bilirubin and bile acids. The effect on mortality seems to be low. BAL systems use viable hepatocytes and are connected to the patient's circulation (Table 1). The concept of artificial liver was developed by Sorrentino in 1956 [42], who proved that liver tissue homogenate could produce urea from ammonia chloride. In 1975 Wolf *et al.* could prove that hepatoma cells placed in the extrafibre space of a hollowfibre cartridge could effectively conjugate bilirubin [43].

For bioreactor cultures various cell sources have been evaluated. Cells used for BAL include human hepatocytes as well as hepatoblastoma-derived cells (C3A cells), but the most used cells are porcine hepatocytes. Porcine hepatocytes are easily cultivated *in vitro* and are available in large quantities but bear the risk of infection (*e.g.* porcine endogenous retrovirus [PERV] or herpes species) and metabolic incompatibility. Human tumour cell lines such as C3A cells can be easily cultivated but have poor liver key functions [44] and a potential tumourigenic ability. Primary human cells meet all the demands of compatibility but usually are not available in appropriate quantities and originate from histologically impaired organs that are not suitable for whole organ transplantation.

The extracorporeal liver assist device (ELAD) uses about 200–400 g of cells of the human hepatoblastoma cell line C3A (derived from HepG2) in modified dialysis-based cartridges. The cells are located in the extracapillary space separated from plasma by a capillary membrane. Prior to entering the bioreactor, the plasma passes an adsorber and a membrane oxygenator. First, clinical applications were performed to demonstrate the safety of the system [45, 46].

The HepatAssist (Circe-Biomed. Inc., Los Angeles, CA, USA) utilizes $5–7 \times 10^9$ cryopreserved porcine hepatocytes that are placed in the device just before clinical use. The function of the bioreactor is supplemented by a column filled with activated charcoal. The patient's plasma is separated using plasmapheresis, transported through the charcoal column, oxygenated and then sent through the bioreactor. A large randomized, controlled multicenter study with a total of 171 patients (86 in the control
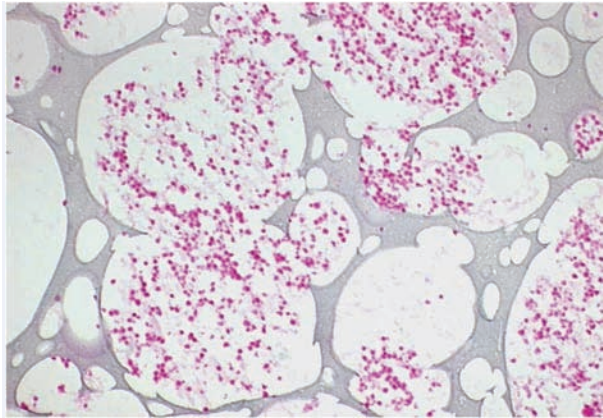
Fig. 2 Rat hepatocytes seeded on a three-dimensional polymeric matrix form a three-dimensional tissue *in vitro* after 3 days in culture.

group and 85 in the bioartificial liver treatment group) was conducted by Demetriou *et al.* [47]. Patients with fulminant/subfulminant hepatic failure and primary nonfunction following liver transplantation were included in the study, demonstrating the safety of the system and an improved 30-day survival in a subgroup. Survival benefit could only be proven for a small group of patients with fulminant liver failure. No PERV could be detected in patients [48].

The MELS (modular extracorporeal liver support) system (Charite, Berlin, Germany) developed by Gerlach *et al.* [5] is based on immobilized hepatocytes [49] in a bioreactor with three independent capillary systems for medium inflow, cell oxygenation and medium outflow. The direct contact of blood cells with the hepatocytes is avoided by a plasmaseparation step and an outflow filtration. A phase I clinical study was performed with the CellModule [50] charged with porcine hepatocytes and 12 patients were treated with human hepatocytes [50]. In 2002 eight patients with ALF could be bridged successfully to transplantation using the MELS system [51]. Another BAL device, the AMC (Amsterdam Medical Centre)-BAL developed by Chamuleau showed significant improvement in hepatic encephalopathy and detoxification in case reports and in one phase I trial [52, 53]. The AMC-BAL uses porcine hepatocytes that are cultivated on a spirally wound polyester fabric. Hollow fibres are used for oxygenation. Preclinical studies suggest that the BLSS (bioartificial liver support system; Excorp Medical, Inc. Minneapolis, Minnesota, USA) impacts the course of

liver failure [54]. The system uses a hollow fibre bioreactor loaded with porcine hepatocytes.

Development of matrix-based hepatocyte transplantation

Over the last years, the injection of liver cell suspensions into anatomic structures such as the spleen [55], the kidney capsule [56] or the peritoneal cavity [57] has been performed in different animal models. Especially, the intraportal hepatocyte injection has been reported to be successful in animal models of metabolic deficiencies [58]. Recently, intraportal hepatocyte injection has been successfully applied on patients with the Crigler-Najjar syndrome type 1 [59]. However, portal hypertension, portal vein thrombosis and pulmonary embolism remain problematic when larger cell numbers are transplanted [60, 61].

Function and differentiation of liver cells are influenced by the three-dimensional organ architecture [62]. This concept led to combine cell transplantation with the application of three-dimensional, highly porous polymeric matrices as a concept of tissue engineering [63] (Fig. 2). It has several advantages when compared to the injection of cell suspensions into solid organs (Table 2). The matrices provide sufficient volume for the transplantation of cell numbers up to whole organ equivalents [64]. Transplantation efficiency could be improved by optimizing shape and composition of the matrices as well as by attaching growth factors and ECMs to the polymeric scaffold [23, 65]. Cell transplantation into polymeric matrices is, in contrast to cell injection into anatomic structures, a reversible procedure because the cell matrix constructs may be removed if so desired. In the future, this concept may even allow the construction of preservable, implantable liver support devices that are available without restrictions. The use of three-dimensional matrices as a carrier for the transplantation of genetically altered cells [66] is also conceivable [67]. Heterotopic hepatocyte transplantation in matrices has been demonstrated in long-term studies [68] (Fig. 3). Nevertheless, initial engraftment rates are suboptimal. In theory, the metabolic situation in patients with hepatic failure or other liver diseases may provide a hepatotrophic stimulus for hepatocytes in heterotopic locations *per se*.

Table 2 Achievements of heterotopic hepatocyte transplantation using 3D matrices

Goal	Comment	Reference
Whole liver mass transplantation		[64]
Coating of matrices with ECM molecules or attachment of growth factors	Increased cell engraftment and function	[23, 65]
Transplantation of genetically altered cells	Correction of metabolic defects	[66, 67]
Co-transplantation of different cell types	Increased hepatocyte survival and proliferation	[73, 74]
Long-term data after hepatocyte transplantation		[68]
Correction of vitamin C deficiency	ODS rat	[64]

ODS, osteogenic disorder Shionogi

However, such hepatotrophic effects could not be observed in animal models of metabolic enzyme deficiencies [69], which are considered the most important future indication for intracorporeal liver support devices. Because optimal transplantation efficiency is a prerequisite for any future clinical application, the improvement of engraftment and the continuous long-term stimulation of hepatocytes in the polymeric matrices are of great interest. Portocaval shunt operation in the recipient is a standard procedure for experimental long-term stimulation of hepatocytes in heterotopic sites [70], but the need for vascular surgery combined with the procedure-specific side effects [71] may reduce its applicability in humans. Selective segmental liver transplantation experiments by Starzl *et al.* [72] revealed that the majority of the hepatotrophic factors in the portal venous blood originate from the pancreatic circulation. Therefore, pancreatic islet co-transplantation seems to be an alternative for the stimulation of hepatocytes in polymeric matrices [73]. Co-transplantation of islets of Langerhans with hepatocytes and portocaval shunt supported engraftment of hepatocytes in polymeric matrices equally well. Islet cell-cotransplantation (ICT) did not interfere with the recipient's glucose metabolism and did not induce hyperproliferative premalignant foci within the transplanted hepatocytes. The technique is therefore an attractive approach towards hepatotrophic stimulation of BAL equivalents [74]. One of the main problems in hepatocyte transplantation – the vascularization and oxygenation of hepatocytes – may be solved by tissue engineering techniques. Recent improvements in the generation of prevascularized bone, fatty tissue and muscle structures are promising and may be transferable to the situation in hepatic tissue engineering [75–79]. In addition, recent data showed the possibility of creating a nanostructure in a capillary pattern by micro-electromechanical system (MEMS) [80]. In such a system a poly-glycol acid film was seeded with human umbilical vascular endothelial cells [81]. The combination of nanoscaffolds with successful prevascularization techniques is a promising tissue-engineered approach to provide sufficient vascularization of artificial tissue constructs for transplantation.

Outlook: future perspective for the development of successful tissue engineering approaches for transplantation

Because there is no sufficient supply of donor organs, and immunosuppression is associated with high morbidity and high costs, there is an urgent need for other therapeutic options. In some cases BAL devices and hepatocyte transplantation have been used with great success, but still have to be improved. Tissue engineering is one of the key techniques to hepatocyte transplantation and to BAL. Better understanding of cell culture techniques is necessary to achieve the goal of building effective liver support devices. First steps toward preformed and functional hepatic tissue with an organ-like microstructure have been made. Several cell types have been investigated for hepatocyte culture: stem cells, oval progenitor cells, mature hepatocytes and
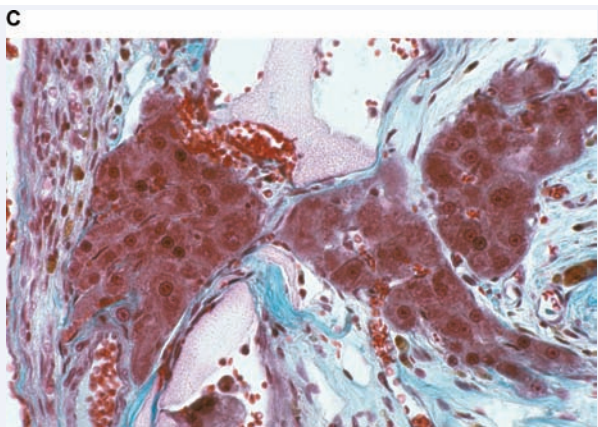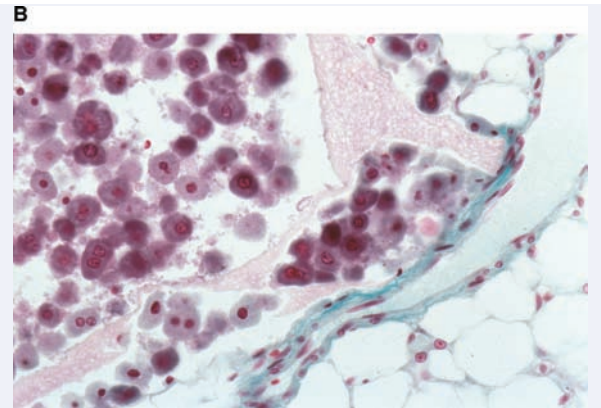
Fig. 3 (**A**) Cell-seeded matrix at implantation between the mesenteric leaves in the peritoneal cavity. (**B**) Microscopic appearance of haematoxylin and eosin stained specimen of a polyvinyl-alcohol matrix seeded with freshly isolated hepatocytes. (**C**) H&E staining of a cell-seeded matrix 3 months after transplantation showed engrafted hepatocytes forming a neo-tissue (magnification 4 x 20).

cells derived from *neo*plastic tissues of the liver. Still many problems have to be solved, but there is hope that cell-based therapies will be the standard therapy for metabolic diseases of the liver one day and that BAL may be as effective for FHF as dialysis is for kidney failure.

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