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Mammalian Hepatocytes as a Foundation for Treatment in Human Liver Failure

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Abstract Technological advances in the separation and culture of mammalian hepatocytes have facilitated the use of these cells as the foundation for either hepatocyte transplantation or hepatocyte-seeded hollow fiber liver assist devices (LAD). To fully appreciate the practical applications of these tissue engineering solutions, it is necessary to understand the types of human liver failure as well as the corresponding animal models. The most immediate application of this type of technology is the treatment of hepatic encephalopathy (HE), an acute and highly fatal complication of fulminant hepatic failure. Although the pathogenesis of HE is unknown, failure of the detoxification function of the liver is accepted as playing an important role in this disorder. Consequently, the assaying and preservation of P450 activity in the grafted cells or in the LAD must be among the main targets of this research. This review explores the problems in hepatocyte transplantation and culture that deserve special consideration and emphasizes the conditions contributing to the in vitro maintenance of phenotypic expression of these cells.

Key words: extracorporeal liver assist device, hepatocyte transplantation, acute liver failure, hollow fiber, P450, hepatocyte culture

Different clinical presentations of liver failure require a variety of therapeutical solutions ranging from a whole organ transplant to extracorporeal devices. These procedures have the potential to use adult hepatocytes as a foundation for treatment in human liver failure. Two approaches based in this type of cellular therapy are discussed here in detail: the intracorporeal grafting of functional cells, i.e., hepatocyte transplantation, and the use of the same type of cells as a component of extracorporeal liver assist devices (LAD). These two techniques may replace and/or complement a current and acceptable treatment of liver failure, namely liver transplantation. In Table I, we present the advantages and disadvantages of liver and hepatocyte transplants and extracorporeal liver assist devices (LADs).

The replacement of a multifunctional organ such as the liver by a man-made (hybrid) device offers a challenge that is rarely duplicated in the field of bioengineering. Yet, it is necessary to determine the type of liver failure before choosing the appropriate therapy. Hepatic failure (HF) can be described as either acute or chronic, based on the span of time that a given agent takes to produce clinical symptoms and/or to perpetuate a clinical condition. In addition, HF is either total or partial, according to the extension of functional impairment.

Chronic total liver failure is now successfully treated by orthotopic liver transplantation [1]. Although the same approach can be used in acute total (fulminant) hepatic failure (FHF), there are higher mortality rates [2]. Availability of cadaveric organs is still a problem, and the concomitant immunosuppressive therapy in patients with HF offers some risks. These situations have encouraged the development of other approaches. An experimental procedure already introduced in the rat model of FHF is heterotopic hepatocyte transplantation [3]. The possibility of transplanted hepatocytes to support temporarily FHF patients depends on both the presence of important detoxification pathways in these cells and the retention of normal blood coagulation products synthesized and stored within the hepatocyte. However, transplanted hepatocytes are subject to immunological rejection [4,5]. As a logical alternative, our laboratory is developing a LAD in which hollow fibers are seeded with xenogeneic or allogeneic hepatocytes which are constantly catabolizing blood

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TABLE I. Advantages and Disadvantages of Liver Transplant, Hepatocyte Transplant, and Liver Assist Device

Advantages	Disadvantages
Liver Transplant	
Orthotopic liver transplant is a well-developed surgical procedure	Requires chronic immunosuppression
Partial liver transplants are feasible, which facilitates the possibility of having a relative as donor	Cyclosporine treatment may produce renal toxicity
Acute rejection is rare	Cadaveric liver availability is a problem
The feasibility of interstate transportation of cadaveric livers was increased by the Wisconsin solution	Expensive
	High mortality FHF
	Retransplants are difficult in FHF
Hepatocyte Transplant	
Possibility of auxillary (heterotopic) transplantation	No established criteria for which is the best site to transplant cells
Minimal surgical procedure	No agreement about critical number of cells to be transplanted (see text)
Banked "cryopreserved" hepatocytes are feasible for use	Some models used for transplanted hepatocytes are not representative of human FHF
Hepatocytes seem to express mainly MHC Class I, but not Class II, antigens	Immunological rejection is possible
	Matrices for cell attachment should be designed carefully (see text)
Liver Assist Device	
Can be incorporated into hemodialysis and/or plasmapheresis procedures	Treatment for HE but not for FHF (chronic hepatic failure) requires transplantation
No major surgical procedure	Could require frequent or prolonged treatment sessions
No immunological rejection Matrices to attach cells are already designed Does not demand a sophisticated surgical center	

toxic products. Similarly, Wolf treated hyperbilirubinemic rats with a hollow fiber LAD seeded with hepatoma cells [6].

Partial liver failure can be either pharmacologically treated by substitution therapy or, in the case of enzyme deficiency disease, by hepatocyte transplantation [7]. The latter seems to offer an alternative to whole organ transplant in treatment of this type of chronic HF.

FULMINANT HEPATIC FAILURE (FHF) AND HEPATIC ENCEPHALOPATHY (HE)

The relationship between FHF and the syndrome of impaired neuromuscular and mental function known as HE has been recognized for many years [8]. HE is considered to be a metabolic process with no evidence of neuronal damage. A plausible course of treatment of HE can be based upon the well-documented capacity of the liver for regeneration and restoration to normal functional and anatomical capacity [9]. Consequently, by replacing impaired liver functions on a temporary basis, some of the proposed treatments *may* sustain life by creating conditions for the liver to regenerate. It follows that in FHF either hepatocyte transplants or the placement of a LAD will not treat liver failure per se but rather the syndrome of HE. This is an

Table II. Metabolic Products With Potential Effects In Hepatic Encephalopathy (9)

Substance	Mode of action
Ammonia	Neurotoxic Interaction with other neurotransmitters Contribute to brain edema
Octopamine	Acts as false neurotransmitter
Mercaptans	Inhibition of Na-K ATPase
GABA	Neural inhibition
Benzodiazepine like substances (endogenous)	Neural inhibitions

important point in the selection of appropriate experimental models of FHF since not all these models reproduce the neurological impairment characteristic of HE.

The pathogenesis of HE is unknown, but two basic hypotheses have been considered. First, in FHF there could be diminished hepatic synthesis of a substance necessary for normal brain function. Carefully designed cross-circulation experiments have not supported this hypothesis [10]. Hence, most investigators favor a second hypothesis: that in FHF there is diminished hepatic metabolism of certain endotoxins, probably gut-derived, which have direct or indirect toxic effects upon CNS synapses [9] or modulate nerve functional inhibitory activities [11]. Table II lists a group of metabolites that have potential for crossing the blood-brain barrier to produce neural inhibition [9]. Controversy exists in defining which of these toxic metabolites is the major agent of HE; if a single agent should be accepted as playing that role, or whether a combination of agents is responsible for that syndrome [12]. All these agents show increased blood values during FHF, but none of them has been definitively proven to be causal for HE.

Experimental findings in animal models, together with the use in humans of experimental drugs known as benzodiazepine receptor antagonists (e.g., RO 15-1788 [flumozenil]), suggest that increased gamma-aminobutyric acid (GABA) is intimately connected with the development of HE, potentiating the action of "normal brain values" of GABA [13]. Using a galactosamine rabbit model of HE, Jones et al. have shown an increased GABAergic tone, although it seems that the substance that rises at the synaptic level is not GABA per se but a substance with a benzodiazepine agonist-like properties [9]. Recently, Maddison et al. reported that taurine is the plasma "GABA-like factor" in the rat model of HE [14].

Assuming that brain benzodiazepine receptors are implicated in the pathogenesis of HE, our laboratory routinely uses the metabolism of diazepam by cultured hepatocytes as an indicator of the cells' P450 activity, and by extrapolation an indicator of the cells' viability. Diazepam is metabolized into three products: temazepam, oxazepam, and nordiazepam. The detection of these metabolites in cultured hepatocytes indicates that the P450 isozymes are actively functioning.

HEPATOCYTE TRANSPLANTATION

Hepatocytes isolated from a variety of animals, either by whole liver collagenase perfusion [15] or by collagenase treatment of partial liver resections [16], can be immediately grafted, or cultured and then grafted. Freshly isolated cells have damaged cell surfaces [17] and may show few of the necessary cell surface receptors for attachment and/or binding of xenobiotics or endogenous toxic products. These cells may function better if they are first cultured, in order to regain their integrity, and then grafted.

Hepatocyte transplantation has been implemented in the jaundice Gunn rat, which is functionally deficient in uridyldiphosphate glucuronyltransferase. In this model, transplant success is assessed by a decrease in total serum bilirubin or by the presence of conjugated bilirubin [18]. Another model of partial chronic HF used for hepatocyte transplantation is the analbuminemic Nagase rat [19].

Transplanted hepatocytes have provided temporary support in various animal models of FHF. In this context, it is evident that many models of FHF (e.g., devascularized liver in the rat or the pig) do not follow the pathogenesis and clinical presentation of HE, which is the major and lethal complication of FHF in humans [13]. Furthermore, there are still no clear evaluations of the minimal number of hepatocytes which must be engrafted to treat HE patients, and it is possible that the beneficial effect of hepatocyte transplantation may be due to trophic factors generated even by few transplanted cells. It has been reported that hepatocyte culture supernatants were as effective as transplanted hepato-

cytes in treating rats with chemically induced liver failure [20].

Further problems for the validation of hepatocyte transplantation are a lack of agreement about 1) the anatomical site for hepatocyte transplants, and 2) what type of matrix (if any) should be used for cell attachment and grafting to insure long term hepatocyte graft survival. Regarding the first subject, intrasplenic [5], intraperitoneal [21], and intrapancreatic [22] grafts have all been implemented with some degree of success. The possibility that the pancreas may provide some trophic effects was demonstrated by experiments in which hepatocyte clusters survived only when transplanted beneath the renal capsule together with pancreatic islets [23].

Hepatocytes are anchorage-dependent cells that require a substrate to thrive. Successful transplantation of cells attached to micro carriers, as demonstrated by Demetriou et al. [19], indicate that such matrices are probably as important for hepatocyte survival in the peritoneal cavity as they are for in vitro survival. These matrices are likely to contribute to better nourishment of the cells by promoting necessary vascularization. Conversely, grafted hepatocytes may not survive when the type of matrix used for their attachment acts as a foreign body. In this situation, unnecessary overgrowth of granulation tissue will probably create a thick collagen capsule that eventually will isolate the grafted cells, contributing to their ischemic necrosis.

Because allogeneic hepatocytes are rejected 4 to 7 days after transplantation (syngeneic hepatocytes are reported to survive for 18 months) [4,5], microencapsulation of allogeneic cells [24] may provide both a substratum for their attachment and a biocompatible membrane to facilitate their immunological isolation.

HOLLOW FIBER HEPATOCYTE CULTURES AS A FOUNDATION FOR LIVER ASSIST DEVICES

Three-dimensional cell cultures on ultrafiltration artificial hollow fibers were introduced by Knazek in 1972 [25]. Hollow fibers are tubular structures with a thin, molecularly discriminating skin (usually on the inner surface). By using hollow fibers with molecular weights (MW) cut-off values of 60,000 to 80,000 daltons, the passage of immunoglobulins is avoided and heterologous hepatocytes can be used without the risk of immunological crossover. Anisotropic, tubular membranes with molecular weights cut off values ranging from 2000 to 120,000 daltons and wall thickness as low as 45 μm or as high as 180 μm have been fabricated for several of our projects by the research division of W.R. Grace. The outer surface of a hollow fiber typically has a "honeycomb" appearance where cells can settle and/or attach. In our hepatocyte tissue culture devices, the hollow fibers are arranged in parallel bundles of a few hundred fibers. Their ends are potted in a thermosetting resin and cut at each extremity to provide a multiperforated "face sheet" (Fig. 1). These bundles are usually encased in cylindrical transparent plastic jackets with side ports for cell seeding. Usually, the provision of high mass transfer rates of tissue culture nutrients or blood nutrients and oxygen (in the case of a device functioning as an artificial liver support) may suffice. The convection of the tissue culture perfusate can be increased by incorporating two sets of capillary bundles to carry the perfusion fluid at different intraluminal pressures [26].

The "state of the art" in hepatocyte culture is such that although limited hepatocyte proliferation in vitro is possible [27] and a prolonged functional viability has been reported by several labs using hormonally defined tissue culture media [28], we still cannot generate primary cultures of hepatocytes that behave as this cell type does in vivo. These remarks should not discourage newcomers to this field since large numbers of viable cells can be obtained from a variety of species. Our laboratory has followed the premise that successful adaptation of hepatocytes to hollow fiber culture conditions should begin with a proper way to separate and isolate these cells [17]. In this context, it should be emphasized that hepatocyte separation, attachment, and successful maintenance of functional viability are intimately connected.

A better understanding of the shortcomings of hepatocyte culture on hollow fibers may greatly improve the design of appropriate LADs. Some methodological limitations require future improvement, e.g., better attachment of these cells to hollow fiber surfaces with preservation of cell shape and polarity, the understanding of what type of hepatocytes should be seeded in these devices, and the design of culture media formulations to maintain hepatocyte phenotypic expression. The latter subject constitutes a moving target which has been discussed prop-

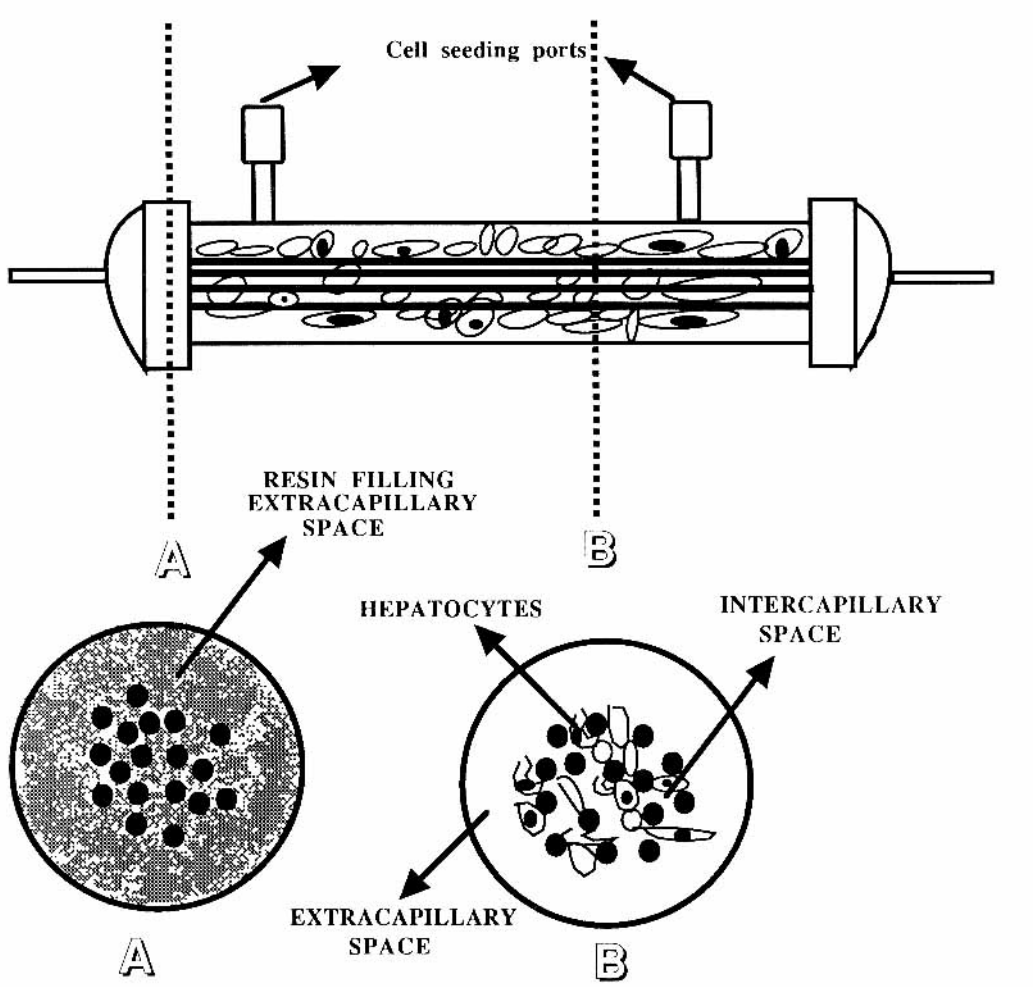

Fig. 1. Schematic of hollow fiber tissue culture chamber. Upper portion depicts the lateral view of the chamber with hepatocytes deposited along the hollow fibers. (**A**) is a cross-section of the end of the chamber with the cross-sections of hollow fibers represented as black filled circles. The extracapillary space is that space between the hollow fiber bundle and the wall of the chamber. The intercapillary space is between the hollow fibers within the bundle. Consequently, the resin fills both the extracapillary and the intercapillary spaces. (**B**) is a cross-section showing the seeded hepatocytes in the intercapillary space, as well as in the extracapillary space. The capillary spaces communicate freely.

erly in the tissue culture literature, whereas the choice of hepatocytes is a subject that has applications to investigators working in hepatocyte transplants or those who are developing LADs. Almost exclusively, LAD design requires proper management of hepatocyte attachment substrates.

Choice of Hepatocytes

Probably one of the most urgent decisions that the designer of LADs will face is the choice of cell to be seeded in these devices (also to be transplanted). Within the possibilities of LAD design, allogeneic or xenogeneic hepatocytes may be used, since the MW exclusion capacity of the hollow fiber internal skin will prevent immunological rejection. In choosing mammalian hepatocytes the investigators should also be aware that the liver lobule is not homogeneous; a division of labor takes place, with periportal cells usually dividing and pericentral cells (area III of Rappaport acinus) displaying sophisticated pathways of cell detoxification. Gumucio et al. have demonstrated that an enrichment of periportal or pericental hepatocytes can be obtained by portal or retrograde collagenase perfusion [29], providing an approach that can be further implemented by elutriation centrifugation. It is also

possible that the use of adult differentiated hepatocytes could be avoided by the use of transfected cells.

Transfer of genetic material into cultured hepatocytes has wide applicability to both hepatocyte transplantation and to the seeding of LADs. A potential drawback, however, is that cultured adult hepatocytes undergo only one or two rounds of division and cannot be passaged. Furthermore, the stable integration of viral sequences used in the transfection must not lead to the complication of malignancy or aberrant gene expression. However, the use of replication-defective retrovirus vectors and the ability to target the recombinant molecule to a specific integration site should overcome this objection to gene transfer. Wolff et al. [30] and Wilson et al. [31] both used replication-defective retrovirus-mediated gene transfer into cultured adult rat hepatocytes and demonstrated stable integration and expression of the transduced genes. Other investigators, to bypass the use of retrovirus vectors, have successfully employed, in vitro, either a calcium phosphate coprecipitation method [32], or a soluble DNA carrier system targeted to hepatocyte-specific asialoglycoprotein receptors [33] to introduce exogeneous DNA. Therefore, transfection of hepatocytes is now within the realm of possibility, as long as the recombinant molecules are carefully constructed to include tissue-specific regulatory sequences. In this way, the foreign DNA will be under the same control mechanisms to which endogenous hepatocellular DNA normally responds.

Hepatocyte Attachment of Hollow Fibers

Hepatocytes attach poorly to and do not spread well on the biopolymers used to manufacture hollow fibers, so the use of substrates as means of attaching the cells is in order. A thin film of collagen on the external surface of the hollow fibers may facilitate cell attachment and spreading leading to maintenance of the cells in vitro [34]. Similar results have been demonstrated when hepatocytes were attached with lectins. This is not surprising since lectins have been used to immobilize cells on nylon fibers for affinity chromatography [35].

Awareness of anatomical relationships of hepatocytes in vivo provides a clue about the best attachment substrates in terms of long term survival. Ideally, conditions for hepatocyte culture in hollow fibers should mimic the in vivo condition by having the cells either in contact with other hepatocytes, with other liver cell epithelial cells, or with liver-specific extracellular matrix (ECM). The importance of cell-cell interactions cannot be minimized, but a LAD device based on hepatocyte co-culture with another cell type complicates the methodology and increases the risk of failure since a second cellular component may proliferate at a higher rate than seeded hepatocytes, which usually do not proliferate in vitro.

Cell co-culturing probably is unnecessary if the substrates used to coat the tissue culture plasticware are representative of that found in the liver. Biomatrix, a mixture of multiple extracellular matrix components isolated from liver should be an ideal substrate [36]. When comparisons have been made between hepatocytes cultured on either Biomatrix or Vitrogen (a commercial mixture of collagen types I–III), those cells on Biomatrix did not exhibit better cell attachment, better glucuronidation (phenol red metabolism), or better protein content [28]. Matrix from Engelbreth-Holm-Swarm (EHS) tumors (Matrigel) introduces components not found in contact with hepatocytes in situ. Claims that this substrate will prolong P450 activity are in dispute, although soluble Matrigel does appear to prolong albumin synthesis [37].

While the mechanism of cell-substrate interaction is poorly understood, it has been hypothesized that matrices contribute to hepatocyte survival in vitro by providing a three-dimensional scaffold for cell attachment. Cells plated at very high densities to maintain their in vivo three-dimensional array have displayed improved survival in the absence of an added substrate [38]. Recently, adult rat hepatocytes cultured as multicellular aggregates (spheroids) in the absence of serum were reported to retain a high albumin producing ability (in contrast to adherent cells) [39]. Therefore, the seeding of hepatocyte aggregates into the external honeycomb structure of hollow fibers in our model of LADs may furnish a successful combination to prolong hepatocyte functional activity in vitro.

It is clear that there are still substantial technological hurdles to be overcome before either hepatocyte transplantation or hepatocyte-seeded LADs can be used in treatment of HE. This endeavor is both exciting and challenging since the successful implementation of these methodologies would produce great clinical benefit.

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