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Hepatic Tissue Engineering for Adjunct and Temporary Liver Support

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59.1 Introduction

Approximately 30,000 patients die each year from end-stage liver disease in the United States. About 80% of these patients have decompensated chronic liver disease, typically caused by alcoholism or chronic hepatitic C infection, and less commonly by a genetic — hepatocellular or anatomic — defect of liver function, or cancer. The other 20% die of acute liver failure (without preexisting chronic liver disease), which has various etiologies, including ischemia–reperfusion injury during liver surgery, acetaminophen poisoning, viral hepatitis, severe sepsis, idiosyncratic drug reactions, etc. Acute liver failure symptoms develop over a period of 6 weeks to 6 months and lead to death in over 80% of the cases, usually from cerebral edema, complications due to coagulopathy, and renal dysfunction. A more severe form of acute liver failure — fulminant hepatic failure — is characterized by a more rapid evolution (2 to 6 weeks).

Orthotopic liver transplantation (OLT) is the only clinically proven effective treatment for patients with end-stage liver disease. The majority of donor livers are obtained from brain-dead cadavers that still possess respiratory and circulatory functions at the time of organ retrieval. Expansion of the donor pool to include living donors, marginal and domino livers, as well as using split livers has been a major

focus of transplant surgeons in the past few years. Although this may alleviate the donor organ shortage, there still remains a great potential benefit to developing alternatives that could be more cost-effective and less invasive, such as adjunct and temporary liver support. These approaches may find applications in the treatment of acute liver failure by allowing endogenous liver regeneration, as well as in chronic liver failure by ameliorating complications arising from the disease. Temporary liver support may also serve as a bridge to OLT by allowing more time to find a better match between donor and recipient or stabilize the patient prior to surgery.

59.2 Adjunct Internal Liver Support

A situation in which the native liver retains some functional capabilities is most amenable to adjunct liver support. The concept of adjunct liver support has been validated by the success of auxiliary partial liver transplantation [1,2]. However, primary nonfunction and vascular complications, for example, portal vein thrombosis, are more frequent with auxiliary partial liver transplantation than with whole liver transplantation. On the other hand, in certain situations, for example, fulminant hepatic failure (FHF), the native liver recovered and the patients could be safely removed from immunosuppressive drug therapy. Hepatocyte transplantation and hepatocyte-based implantable devices are an appealing alternative to auxiliary partial liver transplantation for several reasons (a) several patients could be treated with one single donor liver; (b) the implantation procedure could be performed using less invasive surgery; (c) isolated liver cells can be cryopreserved for long times; and (d) the liver cells could be genetically engineered *in vitro* to upregulate specific functions.

59.2.1 Hepatocyte Transplantation

Hepatocyte transplantation is the simplest form of adjunct internal liver support and has been investigated for over 25 years. In general, the efficiency of engraftment has been found to be quite low and a lag time, which may be as much as 48 h, is necessary before any clinical benefit occurs [3]. Thus, this approach offers an attractive prospect for correcting mostly nonemergency conditions such as inherited metabolic defects of the liver [4]. In early studies the choice of the transplantation site was dictated by accessibility and ease of procedure, as well as by spatial considerations: the pulmonary vascular bed, dorsal and inguinal fat pads, and peritoneal cavity. However, expression of liver-specific functions by transplanted hepatocytes could not be achieved in most of these ectopic sites. A microenvironment resembling that of liver, including a basement substrate to promote hepatocyte anchorage and a venous blood supply mimicking the mechanical and biochemical environment of the hepatic sinusoid is required [5]. The splenic pulp and the host liver itself are now the preferred sites for transplantation of hepatocytes [6]. When implanted into the spleen, hepatocytes may engraft locally or migrate into the liver. Some of the successes with hepatocyte transplantation in experimental animals, although often not very dramatic, have prompted clinical studies. The best results have been obtained in the treatment of specific metabolic disorders; however, except for one case with Crigler–Najjar syndrome type I, there was no detectable long-term function of transplanted human hepatocytes [7].

59.2.2 Implantable Devices

To improve the survival and function of implanted hepatocytes, the latter have been incorporated into biocompatible support materials, effectively constituting an implantable device. There are two major types of implantables devices (a) hepatocytes in open matrices that allow tissue — especially blood vessels — ingrowth from the host, thus leading to integration with the surrounding tissue, and (b) hepatocytes isolated from the surrounding environment in the host by a selective membrane barrier.

In early studies, isolated hepatocytes attached to collagen-coated dextran microcarriers were transplanted by intraperitoneal injection in two rat models of liver dysfunction (a) the Nagase analbuminemic rat, and (b) the Gunn rat, which has and inherited deficiency of bilirubin–uridine disphosphate

glucuronosyltransferase activity causing a lack of conjugated bilirubin in the bile [8]. In both models, microcarriers promoted cell attachment, survival, and function of the transplanted hepatocytes.

Prevascularizing the cell polymer devices in combination with hepatotrophic stimulation have been used to encourage liver tissue regeneration around the implant [9]. Furthermore, materials that biodegrade at controlled rates *in vivo* (such as collagen and poly-lactic-glycolic co-polymers) can be used [10,11], and novel techniques, such as solid freeform fabrication can be used to reproducibly manufacture three-dimensional porous materials of well-defined pore size, distribution and interconnectivity [12,13]. Recently, novel biomaterials that are bioactive as well as resorbable have been developed [14]. For example, biomaterials are being designed to stimulate tissue repair through the release of factors that elicit specific cellular responses, such as cell proliferation, differentiation, and synthesis of extracellular matrix. Thus far, one of the more common approaches is to incorporate growth factors into tissue-engineering scaffolds [15,16]. There is also an interest in using "smart" materials consisting of stimuli-responsive polymers that change their properties in response to changes in the external environment [17].

59.2.3 Encapsulated Hepatocytes

A limitation of hepatocyte-based devices using open matrices is the need to use the host's own cells or at the very least an allogeneic cell source, both of which are very difficult to obtain, which seriously limits the usefulness of these devices. To circumvent this problem, there is great interest in using hepatocytes from xenogeneic sources. Since there is no immunosuppressive regimen that currently exists to prevent rejection of xenografts, hepatocytes have been encapsulated into small microspheres as well as into hollow fibers. In theory, encapsulating with a synthetic, permeable membrane provides a physical separation which protects the cells from the immune system of the host by excluding high molecular weight immunocompetent proteins (e.g., antibodies and complement) as well as leukocytes, while allowing free exchange of nutrients and oxygen. Nevertheless, if the microcapsule causes complement activation after implantation, the breakdown complement products could be small enough to enter the microcapsules and damage the transplanted cells. Initial applications of semipermeable microcapsules contained hemoglobin as blood substitute, enzymes to treat inborn errors of metabolism or absorbents to treat drug overdoses [18]. With advances in genetically engineered cells, microencapsulated cells have been used to remove ammonia in liver failure and amino acids such as phenylalanine in phenylketonuria [19]. Numerous studies have been performed with encapsulated hepatocytes without immunosuppressive drugs. Transplantation of microencapsulated xenogeneic hepatocytes into Gunn rats without immunosuppression reduced serum bilirubin levels for up to 9 weeks before returning to control rat levels, possibly due to the deterioration of the biomaterial [20]. The viability and function of encapsulated hepatocytes is highly dependent on the composition of the hollow fiber material [21]. Better results may be possible if angiogenesis near the capsule surface can be promoted [22], and the formation of a fibrotic layer around the capsule can be avoided [23].

59.3 Extracorporeal Temporary Liver Support

Extracorporeal temporary liver support systems are life-support systems that are analogous in concept to kidney dialysis machines, but specifically designed for liver failure patients. Since the liver has the ability to regenerate, temporary liver support may be sufficient to prevent patient death during the most severe phase of the illness, and allow regeneration of the host liver. The other main purpose of liver support systems is to provide a bridge to transplantation while awaiting a suitable donor. Table 59.1 provides a listing of the various techniques and systems currently being tested in a clinical setting.

59.3.1 Extracorporeal Whole Liver Perfusion

This technique was first used in humans in 1964 [24]. Xenogeneic (pig) livers were used for the first time in human studies in 1965 [25]. Although it fell out of favor due to the development of OLT, extracorporeal

TABLE 59.1 Clinical Trials for Temporary Extracorporeal Liver Support

Device[a]	Configuration	Cell mass and source	Perfusate and treatment protocol	Trial phase	Refs.
Whole liver perfusion					
Whole pig, baboon, or human liver			Whole blood, 5 h median perfusion time, most patients received 1 or 2 perfusions	I/II	[26,36]
Dialysis and filtration systems					
MARS (Teraklin AG, Rostock, Germany)	Albumin-loaded hemofilter, 60-kDa cut-off	None	Whole blood, 12–132 h	I/II	[123,124]
Liver Dialysis Unit (HemoCleanse Technologies, West Lafayette, IN)	Hemodiabsorption across 5-kDa cut-off cellulosic membranes	None	Whole blood, 6 h/day; up to 5 days	FDA approved	[38,39]
Prometheus (Fresenius Medical Care AG, Bad Homburg, Germany)	Hemofilter, 250-kD a cut-off, connected to two adsorber cartridges, in series with conventional dialyser	None	Whole blood, up to 12 h divided into 2 treatments over 2 days	I	[40,125]
Bioartificial livers					
HepatAssist (Circe Biomedical, Lexington, MA)	Hollow-fiber, polysulphone, 0.15–0.20 μm pore size	50 g cryopreserved primary porcine hepatocytes on microcarrier beads[b]	Plasma, 6 h/session; up to 14 sessions	II/III	[126–128]
BLSS (Excorp Medical, Oakdale, MN)	Hollow-fiber, cellulose acetate, 100-kDa cut-off	70–100 g primary porcine hepatocytes	Whole blood, 12 h/session; up to 2 sessions	I/II	[129,130]
MELS (Charité Virchow, Berlin, Germany)	Hollow-fiber (Interwoven, multi-compartment)	250–500 g primary porcine or human hepatocytes	Plasma, continuous up to 3 days	I/II	[131]
ELAD (Vital Therapies, La Jolla, CA)	Hollow-fiber, cellulose acetate, 120-kDa cut-off	100 g human hepatoblastoma C3A cells per cartridge, up to 4 cartridges/device	Plasma, continuous up to 107 h	II (due to resume in 2004)	[132]
AMC-BAL (Hep-Art Medical Devices, B.V., Amsterdam, The Netherlands)	Spirally wound, nonwoven polyester matrix, no membrane	70–150 g[b] primary porcine hepatocytes	Plasma, up to 18 h/session; up to 2 sessions	I	[99,133]
Radial-flow bioreactor (Sant'Anna University Hospital, Italy)	Radial-flow bioreactor	230 g primary porcine hepatocytes	Plasma, 6–24 h treatments, mostly in one session	I/II	[134]
LiverX-2000 (Algenix, Inc., Minneapolis, MN)	Cells embedded in collagen matrix within hollow-fibers	40 g primary porcine hepatocytes per cartridge, 2 cartridges/device	Blood	I/II	[135][c]
Hybrid bioartificial liver (Hepatobiliary Institute of Nanjing University, China)	Polysulfone hollow-fiber cartridge with 100-kDa cut-off combined with adsorption column	100 g primary porcine hepatocytes[b]	Plasma, one 6 h treatment, except one patient with 2 × 6 h treatments	I	[136]

[a] ELAD, Extracorporeal liver assist device; BLSS, bioartificial liver support system; MELS, modular extracorporeal liver system; AMC-BAL, Amsterdam bioartificial liver system; MARS, molecular adsorbent recycling system; FDA, Food and Drug Administration.

[b] Based on 10^8 hepatocytes/g liver tissue.

[c] Clinical data not yet published, reference describes device design.

whole liver perfusion has experienced renewed interest in recent years. Pascher et al. [26] analyzed data from nearly 200 patients from studies conducted from 1964 to 2000, and overall the study concluded that extracorporeal whole liver perfusion was not superior to conventional intensive care treatment approaches.

Early on, a major challenge of this technique was the relatively poor and unstable function of the extracorporeal liver and hemodynamic instability of the patient, which have been improved by using dual vessel (hepatic artery and portal vein) perfusion [27]. Studies in the period ranging from 1990 to 2000 have shown that patients treated with an extracorporeal liver and that survived all eventually received OLT; thus, the extracorporeal liver was potentially effective as a bridge to transplantation, but not as a substitute.

The shortage of human donors provides a strong motivation for the use of xenogeneic livers. Transgenic pigs and immunoabsorption techniques have been used to reduce the effects of hyperacute rejection [28–30]. Given that immune function is severely depressed in acute liver failure patients, this may not be necessary for perfusions lasting 24 to 36 h [31–33]. Borie et al. [34] described an alternative approach to isolate the xenogeneic liver from the host whereby a pig liver is perfused with pig blood in a secondary circuit which is separated from the host's blood by a microporous membrane. Although it appears that immune incompatibilities could be addressed, some of the data suggest that baboon and human livers may be more effective than livers from other species, suggesting an important role for proper matching of the metabolic and physiological activities of the extracorporeal liver and host [35,36].

59.3.2 Dialysis and Filtration Systems

The first attempts at developing devices for temporary and adjunct liver support consisted of nonbiological devices incorporating hemodialysis, hemofiltration, and/or plasma exchange units aimed at removing toxins accumulating in the patient's blood. Charcoal perfusion, the most extensively characterized nonbiological method, showed benefits in various animal models, but no survival benefit was reported in the only one reported randomized clinical trial [37]. Recently, there has been renewed interest in further refining these approaches, with three different systems at various stages of clinical assessment.

The Liver Dialysis Unit (Hemocleanse Technologies, West Lafayette, IN), an approved device in the United States since 1996, is a modified dialysis machine wherein blood is dialyzed against a solution that is continuously recycled through a mixture of sorbents including activated charcoal and an ion-exchange resin. In several small randomized prospectively controlled trials carried out from 1992 to 1998, patients were treated 6 h/day for 1 to 5 days, and the results showed a better outcome with patients with acute on chronic liver failure, although there was no benefit for patients with FHF [38]. The lack of benefit in FHF patients was attributed to the inability to clear strongly protein- or lipid-bound toxins, including bilirubin, endotoxin, and inflammatory cytokines, that are too big to go across the 5 kDa molecular weight cut-off of the dialysis module. A modified version of the device includes a plasma filter module wherein plasma interacts directly with the sorbent particles, thus eliminating this barrier. In preliminary clinical studies, including the plasma filter module resulted in decreases in bilirubin, aromatic amino acids, ammonium, creatinine, and inflammatory mediators such as interleukin-1β [39], but not enough information was available to make conclusions on the overall clinical benefit. A "second-generation" system is currently being designed as kit to convert an existing kidney dialysis into a liver dialysis system, and being touted as a more cost-effective system than its predecessor.

The Prometheus System (Fresenius Medical Care AG, Bad Homburg, Germany) is conceptually very similar to the competing system described above, in that it also includes of two separate modules (a) a high-flux dialyzer that removes water soluble toxins, and (b) a plasma filter module. The latter consists of a large pore (250 kDa) hollow-fiber module that enables albumin along with hydrophobic toxins bound to it to enter a closed loop circuit that contains sorbent materials that strip off the toxins and free up the binding sites on albumin before it is returned to the blood stream. A clinical study in patients with acute-on-chronic liver failure with accompanying hepatorenal syndrome shows that treatment decreased circulating levels of many toxins, such as ammonia, bilirubin, bile acids, etc. although there was no

improvement in the hepatic encephalopathy score [40]. More information on clinical efficacy awaits prospective controlled studies with longer treatment periods.

The Molecular Adsorbent Recirculating System, also known as "MARS" (Teraklin, Rostock, Germany) is a device wherein the patient's blood is dialyzed against an albumin solution, the latter of which is recycled continuously over stripping columns containing various sorbent materials, including activated charcoal [41]. The dialysis membrane has a pore size of 50 kDa, which in principle allows small water-soluble toxins (such as ammonia) to escape, and a hydrophobic coating which allows albumin-bound liposoluble substances in the blood (such as bilirubin and benzodiazepines) to cross the membrane and be picked up by the albumin in the dialysate. In this design, a single module can remove both water-soluble and lipid-soluble toxins. Furthermore, the patient's blood contacts the biocompatible membrane only, and never comes into direct contact with the sorbent materials. Over 3000 patients with various etiologies of liver dysfunction have been treated with this device, and generally show neurological improvement, hemodynamic stabilization, and better hepatic and kidney functions following treatment [42,43]. The small number of controlled trials available for acute liver failure also suggest increased survival in MARS-treated patients [44–47]. Larger multicenter trials in the United States and Europe are currently under way to confirm these very encouraging, yet preliminary, findings. Evidence shows that markers of oxidative stress and systemic inflammation are also reduced after MARS treatment [48].

A meta-analysis published early in 2003 suggests that, overall, artificial liver support systems containing no cells significantly reduced mortality in acute-on-chronic liver failure, although not acute liver failure [49]. Preliminary economic evaluations of such treatments have been performed. In one study with cirrhosis patients undergoing superimposed acute liver injury, cost savings due to reduced liver-disease-related complications more than offset the additional cost of MARS treatment relative to conventional therapy [50]. In another study with patients with acute-on-chronic liver failure due to alcoholic liver disease, cumulative costs per patient in the first year were much higher in the MARS-treated group, although the main explanation appears to be an increase in mean survival time of the patients [51].

59.3.3 Bioartificial Livers

Although such devices are in principle more complex than dialysis and filtration systems, they could provide biochemical and synthetic functions that are not available in the systems containing no cells [52]. The mechanisms of liver failure are not yet well understood and the most critical hepatic functions in patients undergoing liver failure not known; therefore, it is yet unclear whether dialysis and filtration systems, which are likely to be cheaper, will supplant hepatocyte- or cell-based bioartificial livers.

59.3.3.1 Long-Term Hepatocyte Culture Systems

The availability of stable long-term liver cell culture systems that express high levels of liver-specific functions is an essential step in the development of liver-assist devices using hepatocytes. Three types of long-term culture techniques for adult hepatocytes have been used for bioartificial liver development: (a) co-culture of hepatocytes with a "feeder" cell line, such as fibroblasts, (b) three-dimensional network of collagen or other matrix, and (c) hepatocyte aggregates or spheroids. Hepatoma cell lines, which do not require specific substrate configurations, have been used as well. Some of these techniques can be combined; for example, Takezawa et al. [53,54] used thermally responsive polymer substrates to develop multicellular spheroids of fibroblasts and hepatocytes.

59.3.3.1.1 Introducing Non-Parenchymal Cells

Approximately 20 years ago, it was discovered that hepatocytes could be cultured on "feeder or supportive cells" to maintain their viability and function [55]. More recent studies showed that nonhepatic cells, even from other species, could be used. In these culture systems, cell–cell interactions among hepatocytes and cells of another type (rat liver epithelial cells, liver sinusoidal endothelial cells, or mouse embryonic fibroblasts), or "heterotypic interactions," are critical for the expression of hepatocellular functions. The disadvantages of co-culture systems include the potential variability in the cell line used, and the additional work needed to propagate that cell line in addition to attending to the isolation of hepatocytes.

It may be desirable to optimize heterotypic cell–cell interactions in order to maximize the expression of liver-specific functions of the co-cultures. Keeping in mind that cells cultured on surfaces do not usually layer onto each other (except for malignant cancer cell lines), random seeding using a low ratio of parenchymal cells to feeder cells will achieve this goal, but at the expense of using a lot of the available surface for fibroblasts, which do not provide the desired metabolic activity. On the other hand, micropatterning techniques enable the optimization of the seeding pattern of both cell types so as to ensure that each hepatocyte is near a feeder cell while minimizing the number of feeder cells [56]. As a result, metabolic function per area of culture is increased and the ultimate size of a BAL with the required functional capacity is reduced. In prior studies using circular micropatterns, function per hepatocyte increased when the hepatocyte circle diameter decreased, and function per unit area of culture increased when the space occupied by fibroblasts in-between the hepatocyte islands decreased (for a constant cell number ratio of the two cell types).

Various methods for patterning the deposition of extracellular matrix or other cell attachment factors onto surfaces have been developed [57]. Photolithography involves spin-coating a surface (typically silicon or glass) with a ~1 μm thick layer of photo-resist material, exposing the coated material to ultraviolet light through a mask which contains the pattern of interest, and treating the surface with a developer solution which dissolves the exposed regions of photo-resist only. This process leaves photo-resist in previously unexposed areas of the substrate. The exposed areas of substrate can be chemically modified for attaching proteins, etc., or treated with hydrofluoric acid to etch the material. The etching time controls the depth of the channels created. The etched surfaces produced by photolithography can be used to micromold various shapes in a polymer called poly(dimethylsiloxane) (PDMS). The PDMS cast faithfully reproduces the shape of the silicon or glass mold to the micrometer scale, and can be used in various "soft lithography" techniques, including microstamping, microfluidic patterning, and stencil patterning. An infinite number of identical PDMS casts can be generated from a single master mold, which makes the technique very inexpensive. Soft lithography methods can be used on virtually any type of surface, including curved surfaces, owing to the flexibility of PDMS.

59.3.3.1.2 *Hepatocyte Functional Heterogeneity*

In the hepatic lobule, blood flows from the periportal outer region towards the central hepatic vein. Hepatocytes in the periportal, intermediate or centrilobular, and perivenous zones exhibit different morphological and functional characteristics. Spatial heterogeneity in the hepatic lobule is clearly important for some aspects of hepatic function (Figure 59.1a). For example, urea synthesis is a process with high capacity to metabolize ammonia but low affinity for the substrate. Ammonia removal by glutamine synthesis is a high affinity process which removes traces of ammonia which cannot be metabolized by the urea cycle [58]. Co-expression of both enzyme systems would not be productive because the higher affinity process (glutamine synthesis) would be saturated under most operating conditions, leading to a reduced efficiency in ammonia extraction. On the other hand, replicating the functional heterogeneity of hepatocytes in the lobule would likely enhance the performance at the tissue level.

Functional heterogeneity also has important implications in the metabolism of hepatotoxins such as acetaminophen. Acetaminophen is normally degraded by glucuronidation and sulfation reactions which are uniformly distributed along the acinus. After acetaminophen overdose, these processes are saturated and cytochrome P450 activities primarily located in the centrilobular region metabolize significant amounts to toxic metabolites causing oxidative stress and protein cross-linking. Although these metabolites can be detoxified by glutathione-dependent reactions, centrilobular hepatocytes do not have an efficient glutathione recycling system, and as a result are the main target of acetaminophen-induced hepatotoxicity. Repeat exposure to incremental doses of acetaminophen increases the tolerance to hepatic damage by partially shifting the expression of cytochrome P450 towards the periportal region [59], which has the most active glutathione recycling metabolism in the liver [60].

The maintenance of functional heterogeneity in the liver is dependent on several factors, including gradients of hormones, substrates, oxygen, and extracellular matrix composition, although the relative importance of each one of these factors is currently unknown. In one study where hepatocytes were

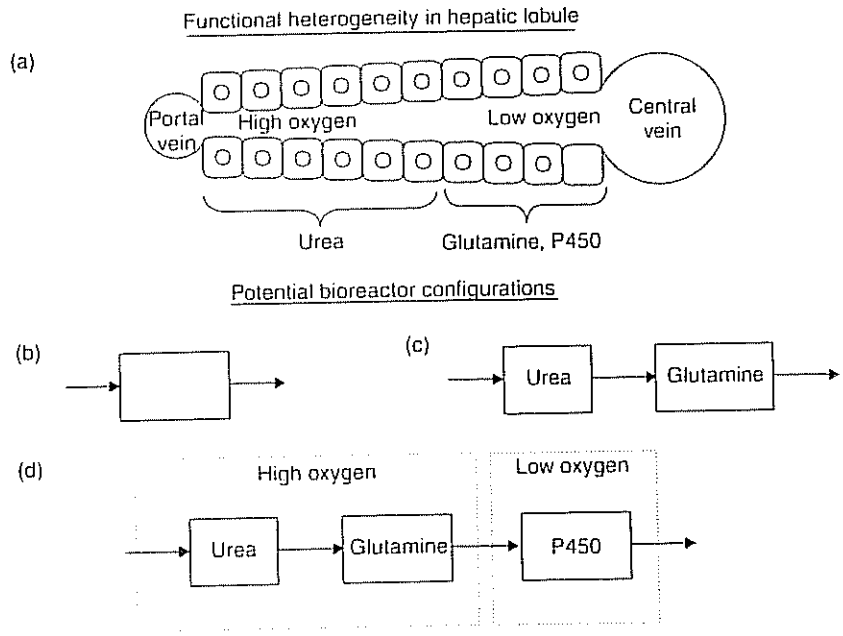


FIGURE 59.1 Potential bioreactor configurations in a bioartificial liver. (a) *In vivo* distribution of hepatocellular functions. (b) Single unit optimized to perform all functions. (c) Two subunits, the first one optimized for ammonia conversion to urea, a high capacity but low affinity process, and the second one optimized for ammonia conversion to glutamine, a high affinity process that scavenges ammonia not metabolized in the first subunit. (d) Three subunits, the first two designed to clear ammonia under high oxygen tension, and a third subunit operating at lower oxygen tension that is optimized for efficient P450 detoxification pathways.

chronically exposed to increasing oxygen tensions within the physiological range of about 5 mmHg (perivenous) to 85 mmHg (periportal), urea synthesis increased about 10 fold, while P450IA1 activity decreased slightly and albumin secretion was unchanged [61]. These data suggest that by creating environmental conditions which emulate certain parts of the liver sinusoid, it is possible to modulate hepatocyte metabolism in a way that is consistent with *in vivo* behavior. Spatial control of the layout of the cells in the device may be achieved using micropatterning and microfabrication techniques [62,63], or using separate bioreactor modules that are optimized to perform a subset of hepatocellular functions, as illustrated in Figure 59.1b–d.

It is also possible to profoundly affect the expression of liver-specific functions by hepatocytes by changing a number of environmental conditions in the bioreactor environment. For example, urea synthesis dramatically increases with increasing oxygen tension while cytochrome P450 decreases [64]. Amino acid supplementation to human plasma increases urea and albumin synthesis, as well as cytochrome P450 activities [65]. Co-culture with mouse 3T3 cells also increases albumin and urea secretion to levels which exceed *in vivo* rates severalfold [66,67]. While albumin and urea secretion decrease at higher fluid shear rates, the latter tend to increase cytochrome P450 detoxification rates, at least in the short term [68]. Sophisticated optimization techniques that can tackle the large number of adjustable environmental variables may be helpful for optimizing the bioreactor environment [69,70]. Since varying one specific environmental condition increases the expression of liver-specific functions many times, it is reasonable to assume that optimization of several such parameters simultaneously may yield an order of magnitude or more in improvement.

59.3.3.1.3 Pre-Conditioning Hepatocytes Prior to Plasma Exposure

Rat hepatocytes which are seeded and maintained in standard hepatocyte culture medium and then exposed to either rat or human plasma become severely fatty within 24 h with a concomitant reduction in

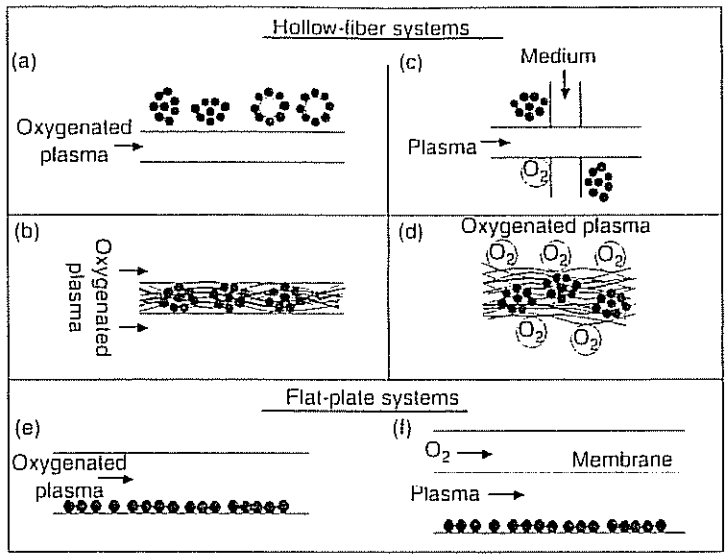


FIGURE 59.2 Most common bioreactor design for bioartificial livers. (a) Hepatocyte aggregates or seeded on microcarriers are placed on the outside of hollow fibers. Oxygenated plasma is flown through the hollow fibers. (b) Hepatocyte aggregates in a supporting matrix are inside hollow fibers and oxygenated plasma is flown outside the hollow fibers. (c) Similar to panel A, although separate hollow fibers are used to deliver hepatocyte culture medium and oxygen into the system. Circle with O_2 is a hollow fiber perpendicular to the plane of the paper. (d) Hepatocyte aggregates are in a supporting matrix next to hollow fibers that deliver oxygen. Oxygenated plasma is flown in the space outside of the hollow fibers and percolates through the matrix–hepatocyte network. (e) Hepatocytes are seeded as a monolayer on the bottom surface of a flat plate and placed within a parallel-plate flow chamber. Oxygenated plasma is flown directly above the cells. (f) System is similar to panel E, except that oxygen is delivered through a permeable membrane directly above the flow channel with the hepatocytes.

liver-specific functions. Thus, plasma appears to be a rather inhospitable environment to the hepatocytes, yet it is clear that hepatocytes must be made to tolerate it for the concept of bioartificial liver to become reality. Supplementation of human anticoagulated plasma with hormones and amino acids (to bring those metabolites to levels similar to that found in standard hepatocyte culture medium) eliminate intracellular lipid accumulation and restores albumin and urea synthesis as well as P450-dependent detoxification [71,72]. However, direct supplementation of plasma, especially with respect to the high levels of hormones used, would be very costly and pose a health risk to the patient.

In prior studies, we have shown that the culture conditions used prior to placing the hepatocytes in contact with human plasma as well as during plasma exposure, can dramatically affect hepatocellular metabolism. For example, hepatocytes cultured in standard hepatocyte culture medium containing supra-physiological levels of insulin become fatty once they are exposed to plasma, and this can be prevented by "preconditioning" the cells in a medium containing physiological levels of insulin [64]. Direct amino acid supplementation to the plasma also increased both urea and albumin secretion rates by the hepatocytes. Thus, a combination of preconditioning and plasma supplementation can be used to upregulate liver-specific functions of hepatocytes during plasma exposure.

59.3.3.2 Hepatocyte Bioreactor Designs

The most popular bioreactor designs are shown in Figure 59.2 and discussed in greater detail below. Most devices tested clinically consist of hollow fiber cartridges containing either porcine hepatocytes or human hepatoblastoma cells. In most cases, cells are loaded into the extraluminal compartment and patient plasma or blood is perfused through the fiber lumens [73–75]. Similar hollow fiber cartridges have also been used in animal studies with hepatocytes seeded inside the fibers and the plasma flowing over the outer surface

of the fibers [76,77]. Because of the relatively large diameter of the fibers as well as transport limitations associated with the fiber wall, these systems are prone to substrate transport limitations [78].

59.3.3.2.1 *Minimum Cell Mass and Functional Capacity*

The cell mass required to support an animal model of hepatic failure has not been systematically determined. Prior studies have shown significant improvements in various parameters using as low as 2 to 3% of the normal liver mass of the animal [79,80]. Devices that have undergone clinical testing have used 6×10^9 to 1×10^{11} porcine hepatocytes [81,82] or 4×10^{10} C3A cells [83]. Recently, in an experimental pig model of hepatic failure, treatment with a bioartificial liver containing 6×10^8 pig hepatocytes (about 3 to 5% of the liver mass) significantly improved survival [84]. Recently, there have been efforts to improve cell viability in large-scale devices. Hepatocytes have been transfected with an antiapoptotic gene (nitric oxide synthase) or exposed to an antiapoptotic drug (ZVAD-fmk) to increase their resistance to what appears to be mainly hypoxic injury [85,86].

Clinical improvements have also been seen in hepatocyte transplantation studies using less than 10% of the host's liver mass. Intrasplenic transplantation of 2.5×10^7 allogeneic rat hepatocytes (about 5% of the rat liver mass) prolonged the survival, improved blood chemistry, and lowered blood TGF-β_1 (an inhibitor of hepatocyte growth) levels in anhepatic rats [87]. In another study using reversibly transformed human hepatocytes, 50×10^6 cells were injected intra-splenically into rats subjected to a 90% hepatectomy [88]. In a recent study on humans with acute liver failure, intrasplenic and intra-arterial injections of human hepatocytes (ranging from 10^9 to 4×10^{10} per patient, i.e., 1 to 10% of the total liver mass) transiently improved several blood chemistry parameters and brain function after a lag time of about 48 h, but did not improve survival [3]. The lag time before any benefit is observed may reflect the time required for the engraftment of the cells in the host liver. Better survival of the injected cells may be possible if the cells are seeded in prevascularized polymeric scaffolds [89]. The relatively low number of hepatocytes needed to effect a therapeutic benefit may be due, in part, to the fact that the exogenously supplied hepatocytes may aid the regeneration of the native liver [79].

Assuming that the minimum cell mass necessary to support a patient undergoing acute liver failure is about 5 to 10% of the total liver weight, this yields a bioartificial liver containing about 10^{10} cells. Designing this system with a priming volume not exceeding about 1 l is still a daunting challenge. Knowing which functions are most critical would help to rationally improve the efficacy of bioartificial liver systems and dramatically reduce the minimum therapeutic cell mass. For example, it is well known that hepatocytes exhibit a metabolic zonation along the acinus [61]. Periportal and centrilobular hepatocytes express high levels of urea cycle enzymes and low levels of glutamine synthetase while pericentral hepatocytes are the opposite [58]. Another example is the reduction in albumin synthesis during the acute phase response, a process which may help sustain the increased level of acute phase proteins [90].

59.3.3.2.2 *Oxygen Transport Issues*

In a normal liver, no hepatocyte is further than a few micrometers from circulating blood; thus, transport by diffusion only has to occur over very short distances. Although oxygen diffusivity is an order of magnitude greater that that of many other small metabolites (e.g., glucose and amino acids), it has a very low solubility in physiological fluids deprived of oxygen carriers. Thus, it is not possible to create large concentration gradients which would provide the driving force for rapid oxygen transport over long distances. This, in addition to the fact that hepatocytes have a relatively high oxygen uptake rate [91,92], makes oxygen transport the most constraining parameter in the design of BAL devices.

Oxygen transport and uptake of hepatocytes has been extensively studied in the sandwich culture configuration in order to obtain the essential oxygen uptake parameters needed in the design of bioreactor configurations [93]. The maximum oxygen uptake rate of cultured rat hepatocytes was measured to be about 13.5 pmol/sec/μg DNA, which is fairly stable after the first day in culture and for up to 2 weeks. Interestingly, the oxygen uptake was about twice in the first day after cell seeding, presumably because of the increased energy requirement for cell attachment and spreading. This may need to be taken into account when seeding hepatocytes into a BAL. Oxygen uptake was not sensitive to the oxygen tension

in the vicinity of the hepatocytes up to a lower limit of about 0.5 mmHg, below which oxygen uptake decreased, suggesting that it becomes a limiting substrate for intercellular hepatocyte metabolism. Since oxygen is essential for hepatic ATP synthesis, a reasonable design criterion is that the oxygen tension should remain above ~0.5 mmHg.

Based on these parameters, it is possible to estimate oxygen concentration profiles in various bioreactor configurations based on a simple diffusion–reaction models assuming Michaelis–Menten kinetics. Generally, one can estimate that the maximum thickness of a static layer of aqueous medium on the surface of a confluent single hepatocyte layer is about 400 μm [94]. Calculations on oxygen transport through hepatocyte aggregates suggest that even a relatively low density of cells (10^7 cells/cm^3) cannot have a thickness exceeding about 300 to 500 μm. At cell densities of 10^8 cells/cm^3, which is similar to that found in normal liver, that thickness is only 100 to 200 μm.

59.3.3.2.3 Hollow-Fiber Systems

The hollow fiber system has been the most widely used type of bioreactor in BAL development [77,95]. The hollow fiber cartridge consists of a shell traversed by a large number of small diameter tubes. The cells may be placed within the fibers in the intracapillary space or on the shell side in the extracapillary space. The compartment which does not contain the cells is generally perfused with culture medium or the patient's plasma or blood. The fiber walls may provide the attaching surface for the cells and/or act as barrier against the immune system of the host. Microcarriers have also been used as a way to provide an attachment surface for anchorage-dependent cells introduced in the shell side of hollow fiber devices. There are many studies on how to determine optimal fiber dimensions, spacing, and reactor length based on oxygen transport considerations [78].

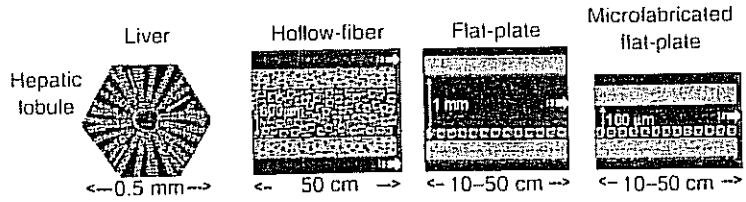
One difficulty with the hollow fiber configuration is that interfiber distances, and consequently transport properties within the shell space, are not well controlled. Thus, it may be advantageous to place cells in the lumen of small fibers because the diffusional distance between the shell (where the nutrient supply would be) and the cells is essentially equal to the fiber thickness. In one configuration, hepatocytes have been suspended in a collagen solution and injected into the lumen of fibers where the collagen is allowed to gel. Contraction of the collagen lattice by the cells even creates a void in the intraluminal space, which can theoretically be perfused with hormonal supplements, etc. to enhance the viability and function of the cells, while the patient's plasma flows on the shell side. Because of the relatively large diameter of the fibers used as well as transport limitations associated with the fiber wall, these systems have been prone to substrate transport limitations.

To improve oxygen delivery, novel designs using additional fibers which carry gaseous oxygen straight into the device have been used [96,97]. Using this approach, Gerlach et al. [82] were able to demonstrate that hepatocytes could express differentiated functions over several weeks. Using a device consisting of hepatocytes seeded onto a woven polyester substrate with integrated hollow fibers for oxygen supply, Flendrig et al. [96,98] showed that the survival time of pigs undergoing total hepatic ischemia was significantly increased over the control group; more recently, this device was successfully used to treat seven acute liver failure patients, of which six were bridged to a transplant and one spontaneously recovered [99].

59.3.3.2.4 Parallel Plate Systems

An alternative bioreactor configuration is based on a flat surface geometry [80,93,100,101] where it is easier to control the internal flow distribution and ensure that all cells are adequately perfused. Its main drawback is that it is difficult to build a system which contains a sufficient cell concentration (Figure 59.3). For example, a channel height of 1 mm would result in a 10 l reactor to support 20×10^9 hepatocytes cultured on an area of 10 m^2. For a liver failure patient who is probably hemodynamically unstable, it is generally accepted that the priming volume of the system should not exceed 1 l.

The volume of the device in the flat-plate geometry can be decreased by reducing the channel height (Figure 59.3). However, this forces the fluid to move through a smaller gap, which rapidly increases the drag force (shear stress) imparted by the flow on the cells. Recent data suggest that hepatocyte function decreases significantly at shear stresses >5 dyn/cm^2 [102]. To reduce the deleterious effects of high shear,



	Liver	Hollow-fiber	Flat-plate	Microfabricated flat-plate
Total cell number	10^{11}	2×10^{11}	2×10^{10}	2×10^{10}
Cell density (per ml)	10^{8}	10^{7}	10^{6}	10^{7}
Total surface area for transport (m^2)	100	2	10	10
Fraction of cells at surface	>90%	<10%	100%	100%

FIGURE 59.3 Comparison between popular hepatocyte bioreactor configurations.

it may be possible to use grooved surfaces where cells lodge and are less exposed to the shear stress, as previously done for blood cells [103,104]. Cells lodge inside the grooves where they are less exposed to the shear stress, which allows for faster flow without causing cell damage. The grooves may on the other hand significantly increase the fluid hold-up volume [105].

In an attempt to provide to cells adequate oxygenation and protection from shear in perfused bioreactors, gas-permeable membranes as well as membranes separating cells from plasma have been incorporated into the flat-plate geometry. Recently, a flat-plate microchannel bioreactor where cells directly contact the circulating medium was developed [93]. The channel is closed by a gas-permeable membrane on one surface, which decouples oxygen transport from the flow rate in the device. Comparing this with a similar flat-plate design where a nonpermeable glass surface is substituted to the membrane, internal membrane oxygenation removed the oxygen limitations that occur at low volumetric flow rates [105]. De Bartolo et al. [80] incorporated two membranes into the flat-plate geometry. The first membrane is gas-permeable and minimizes the oxygen transport limitations in the system. The second membrane separates cells from plasma and adds a significant barrier to the transport of protein-bound toxins that need to be processed by the cells [78]. Others have reported the design of a radial flow bioreactor with an internal membrane oxygenator for the culture of hematopoietic cells [106]. Based on a theoretical analysis, the proposed design would have removed oxygen transport limitations in the bioreactor, but no experimental data were shown.

59.3.3.3 Potential Sources of Cells for Bioartificial Livers

Although several technical difficulties remain to be addressed with respect to the design of implantable and extracorporeal liver-assist devices, clearly a major hurdle for both approaches is the procurement of a sufficient number of cells that are required to achieve a therapeutic effect. Human hepatocytes appear to be the "natural" choice for hepatocyte transplantation, internal and external liver assist devices, however, they are scarce due to a competing demand of OLT. Whether adult human hepatocytes can be induced to replicate *in vitro* and the daughter cells express high levels of liver-specific functions remains to be shown. Human hepatocyte cell lines have been developed via spontaneous transformation [107], as well as via retroviral transfection of the simian virus 40 large T antigen [108]. Recently, a novel technology which uses a reversible transformation strategy with the SV40 T antigen and Cre–Lox recombination was used to grow human hepatocytes *in vitro* [88]. These cells, when transplanted into the spleen of 90% hepatectomized rats, improved biochemical and clinical parameters. In bioartificial liver devices tested so far, the only human cells used have been the cancer-derived C3A line [74,83]. However, one study suggests that C3A cells have lower levels of P450IA1 activity, ammonia removal, and amino acid metabolism that adult porcine hepatocytes [109]. Furthermore, when using immortalized human cell lines, there are concerns with the possibility of transmission of tumorigenic products into the patient. Xenogeneic

hepatocytes offer no risk of transmitting malignancies to the patient, but pose other problems, including the risk of hyperacute rejection [110], transmission of zoonoses [111], and potential mismatch between xenogeneic and human liver functions. The first two could be addressed by dedicated breeding programs of transgenic animals. On the other hand, little is known about the third factor.

Although no one has achieved the goal of generating a safe, fully functional yet clonal, immortalized, or genetically engineered human cell that can be substituted for primary hepatocytes, a new promising avenue is the discovery of liver stem cells. The existence of hepatic stem cells was hypothesized over 40 years ago [112], and recent data suggest that there are stem cells present within [113–115] as well as outside the liver [116], which can differentiate into fully mature hepatocytes. *In vitro* studies suggest the presence of a subpopulation of small hepatocytes in rat liver with a high proliferative potential [117]. Three independent studies in rats, mice, and humans have shown that a major extrahepatic source is stem cells of the bone marrow which may take part in normal tissue renewal as well as in liver regeneration after severe experimentally induced hepatic injury [116,118,119].

59.3.3.4 Techniques for Preservation of Hepatocytes and Liver Cells

The development of optimal preservation protocols for hepatocytes that enable the storage and ready availability of cells for BALs, has been the subject of several studies. Hepatocytes have been cryopreserved shortly after isolation as well as after culture for several days. Compared to isolated cells, cultured hepatocytes exhibit greater resistance to high concentrations of the cryoprotective agent dimethyl sulfoxide, as evidenced by preservation of cell viability, cytoskeleton, and function. Based on experimental and theoretical studies, cooling rates between 5 and 10°C/min caused no significant decrease in albumin secretion rate compared to control, unfrozen, cultures [120,121]. There have been attempts to store hepatocyte cultures in various solutions used for cold storage of whole donor livers. One study showed that cultured hepatocytes maintained at 4°C lose significant viability after a few hours of cold storage, but that addition of polyethylene glycol significantly extends functionality and survival [122]. Interestingly, the use of the University of Wisconsin (UW) solution, currently the most widely used solution for cold organ storage, has not performed better than leaving the cells in standard hepatocyte culture medium. It is conceivable that the UW solution mediates its effect by prolonging the survival of nonparenchymal cells. It is hoped that further improvements in preservation solutions will enable the storage of BAL systems, as well as lengthen the useful cold storage time of whole livers for transplantation.

59.4 Summary

The severe donor liver shortage, high cost, and complexity of orthotopic liver transplantation have prompted the search for alternative treatment strategies for end-stage liver disease that would require less donor material, be cheaper, and less invasive. Adjunct internal liver support, which may be provided via auxiliary partial liver transplantation or hepatocyte transplantation, is most suitable for cases where the native liver retains some functional capabilities, and may be a cure for patients who suffer from specific metabolic disorders. Acute liver failure patients will benefit most from extracorporeal temporary liver support, which can be used as a bridge to transplantation, or as a means to support the patient until its own liver regenerates. Currently, there are three approaches for extracorporeal temporary liver support: extracorporeal liver perfusion, dialysis and filtration systems containing no cells, and bioartificial livers. Dialysis and filtration systems, which do not contain any living cells, are ahead with respect to clinical testing and gaining regulatory approval. A concern with such systems is that their efficacy may be limited due to the lack of metabolic and protein synthetic activities which are normally present in the liver. Bioartificial livers containing liver cells would overcome this limitation, and have passed the "proof of principle" test in preclinical and clinical studies, although tangible clinical benefits have not yet been demonstrated. Important unresolved issues for bioartificial livers and extracorporeal liver perfusion are the identification of a reliable cell/tissue source and a better understanding of metabolic and immune incompatibilities arising from the use of allogeneic and xenogeneic liver cells. Ultimately, several temporary

and adjunct treatment approaches may be available, and the best choice may depend on the etiology of liver failure in each individual patient.

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