Liver, Bio-Artificial

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INTRODUCTION

The liver is one of the most complex and metabolically active organs in the body and performs many detoxification and protein synthetic functions that are essential to life. Extracorporeal bioartificial liver (BAL) systems consisting of functioning, viable hepatocytes may provide temporary support for patients with acute hepatic failure and save the lives of patients awaiting orthotopic liver transplantation (OLT).

In the past few years, we have seen several clinical studies testing the efficacy of BAL devices. These preliminary studies have provided some promising results, although current-generation liver-assist devices have not yet demonstrated sufficient efficacy and reliability for routine use. Some of the issues that the field of BAL development continues to wrestle with are: 1) how to support a large cell mass without substrate limitations so that cells function with maximum efficiency; 2) how to maintain high levels of stable long-term liver-specific function in an artificial (and potentially inhospitable) environment; and 3) how to minimize the priming volume (or dead space) that must be filled by blood or plasma from the patient. It is becoming clearer every day that a more fundamental understanding of the effect of environmental parameters on hepatocellular function, as well as host-BAL interactions, is necessary before the concept of BAL becomes a reality available at reasonable cost. This article describes the tissue-engineering principles and critical technologies that are relevant to the design of BAL devices, and provides future directions for the development of the next generation of BAL support systems.

CLINICAL RELEVANCE

The Clinical Problem

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—hepatocellu-lar 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 (2–6 wk).

Orthotopic liver transplantation is the only clinically proven effective treatment for patients with end-stage liver disease. Acute liver patients have the highest priority for donor livers; however, because acute liver failure is an uncommon condition, it only accounts for 12% of OLTs.[1] The latest data from the United Network for Organ Sharing, the organization that keeps track of organ transplants in the United States, show that the number of liver transplants performed yearly has increased at a constant rate of 240/year since 1992, to reach 4950 in 2000 (Fig. 1). However, the number of patients dying while on the waiting list has increased exponentially at a rate of 16–17% per year. As of August 31, 2002, a staggering 17,436 patients were on the liver transplant waiting list. Currently, 90% of the donated livers are transplanted; thus, any significant increase in the donor supply can only be achieved through dramatic improvements in the methods of organ procurement, as well as expansion of donor criteria.

The majority of donor livers are obtained from brain-dead cadavers that still possess respiratory and circulatory functions at the time of organ retrieval. A large pool that remains largely untapped consists of nonheart-beating cadavers.[2] In 2001, only 3% of the cadaveric livers were from nonheart-beating donors, although it is estimated that the potential may be on the order of 250,000 annually.[3] Such livers, however, are prone to primary nonfunction posttransplantation, and much more research is needed to develop techniques that would recondition or repair these organs to make them acceptable donors. Living-related donor transplantation, which involves
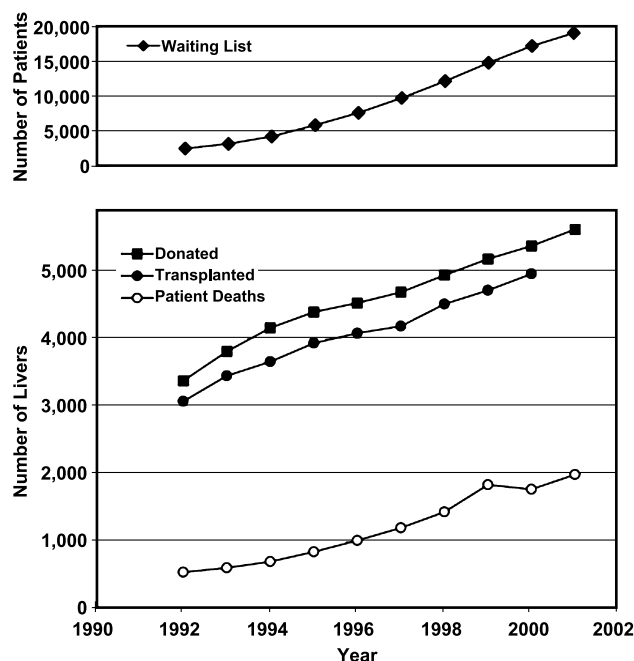
Fig. 1 Evolution of the supply and demand for donor livers in the United States. (Data from Ref. [1].)

using a graft obtained by means of a partial hepatectomy from a living donor (usually a parent or relative), is another promising alternative. Nevertheless, split- and living-liver donor techniques represent only about 3% of the total number of transplants performed in the United States,[4] and are inherently limited because this procedure represents a significant risk for the donor.

All the aforementioned approaches to expand the donor pool are being actively pursued, and have the advantage that they do not require extensive regulatory review, thus shortening the time from concept to practice. Nevertheless, the extent of the problem is such that other alternatives to OLT are desperately needed. Some of the recent and more innovative approaches currently being explored are: 1) xenotransplantation; 2) hepatocyte transplantation; and 3) temporary liver support.

Xeno (cross-species transplantation)

The transplantation of pig or baboon livers into humans has had several failed attempts in the recent past. For this approach to succeed, patients must first overcome hyperacute rejection, a very rapid immune response ($\sim$1 hour) that is characteristic of xenotransplantations, and must actively develop microchimerism, an interaction of two coexisting cell populations leading to the down regulation of both recipient and graft immune systems.[5] Another

major concern is the potential transmission of infectious agents across species.[6] Recent advances in genetic engineering may allow the modification of the immune response, and sophisticated breeding programs may provide animal breeds devoid of zoonotic diseases. These advances may eventually make xenogeneic livers a viable option for use as a bridge to transplantation, or possibly even as a permanent graft.

Hepatocyte transplantation

Transplantation of liver parenchymal cells (hepatocytes) offers the possibility of treating several patients with one single donor liver. The survival and function of implanted hepatocytes can be improved by incorporating the cells into implantable devices such as microcarriers (beads 200–300 μm in diameter that the cells can attach to), hydrogels, and other polymeric matrices. The most suitable implantation sites appear to be those that provide a microenvironment resembling that of liver, i.e., with a substrate that promotes hepatocyte attachment and a venous blood supply network, such as the splenic pulp and the host liver itself. Furthermore, permanent incorporation may require that the liver have significant preexisting damage and that the transplanted cells have a competitive advantage (e.g., they proliferate faster) v. those in the host's liver.[7] Studies so far suggest that the efficiency of engraftment is quite low and that a lag time (which may be as long as 48 hours) is necessary before any clinical benefit occurs.[8] (A 48-hour lag time may be too long in a rapidly deteriorating patient.) On the other hand, this approach offers an attractive prospect for correcting non-emergency conditions such as inherited metabolic defects of the liver.

Other implantable devices, such as capsules and hollow fibers, have been used to protect hepatocytes from the immune system of the host. Some encouraging results have been obtained over a period of a few weeks after implantation, but not beyond, possibly due to deterioration of the biomaterial.[9,10] A common recurrent problem is the presence of a foreign-body reaction against the capsule material itself, leading to the generation, over a period of days to weeks, of a fibrotic layer around it, compromising metabolite transport between the implanted cells and the surroundings. Besides improvements in the biocompatibility of the material, one avenue that may improve the function of these devices is the use of materials or factors that promote the growth of blood vessels near the surface of the capsule.

Temporary liver support

Temporary liver support is a logical option for patients with acute liver failure where the host's liver has the

potential to fully recover, as well as for use as a bridge to transplantation. The concept of temporary liver support is best exemplified by results obtained with auxiliary partial liver transplantation (APLT). Auxiliary partial liver transplantation, currently the only reliable method of temporary liver support, consists of implanting a functioning liver without removing the host's native organ. Auxiliary partial liver transplantation has been used to treat specific inborn liver disorders as well as acute fulminant hepatic failure. In certain cases of hepatic failure, the native liver recovered and the auxiliary liver could be safely removed, hence eliminating the need for lifelong immunosuppression.[11] Indeed, one of the greatest appeals of temporary liver support is the possibility of spontaneous recovery without the need for transplantation. This would free up donor livers as well as eliminate the need for lifelong immunosuppression that is required by all transplant recipients, translating into significant cost savings for the health care system. Temporary liver support may also be used to maintain the patient's life during the long wait for a donor, as well as prevent liver failure-related complications that may make the patient too ill to tolerate a liver transplantation procedure.

Auxiliary partial liver transplantation depends on the availability of donated livers; there is thus a need to develop an off-the-shelf device akin to the kidney dialysis machine. Early devices for temporary liver support contained no cells (i.e., charcoal adsorption columns), and could not demonstrate therapeutic effectiveness.[12] Nevertheless, there remains an interest in improving this methodology. Because the liver provides a myriad of detoxifying as well as protein synthetic functions, efforts in this area are mostly dedicated to the development of bioartificial systems incorporating live liver cells.

CELL SOURCE AND THERAPEUTIC CELL MASS

What Is the Therapeutic Cell Mass?

The cell mass required to support a patient during acute liver failure has not been systematically determined. Devices that have undergone clinical testing have used 5×10^9–6×10^{10} porcine hepatocytes[13,14] or 4×10^{10} human hepatoma cells.[15] In a more recent study on humans with acute liver failure, intrasplenic and intraarterial injections of human hepatocytes ranging from 10^9–4×10^{10} per patient (i.e., 1–10% of the total liver mass) transiently improved brain function and several blood chemistry parameters, but not survival.[8] Results from animal studies also suggest that relatively few hepatocytes (between 2% and 10% of the host's liver)

are needed to effect a therapeutic benefit. This may be due in part to the fact that the exogenously supplied hepatocytes may aid the regeneration of the native liver. Current consensus is that the minimum cell mass necessary to support a human undergoing acute liver failure is about 5–10% of the total liver weight, or about 10^{10} cells.

Cell Source and Replication of Hepatocytes in Culture

Primary (i.e., directly isolated from an organ) human hepatocytes are the natural choice for the BAL. However, they are scarce due to the competing demand for livers for OLT. Human hepatocyte lines have been generated by the spontaneous transformation of long-term cultures of adult hepatocytes, as well as by a reversible transformation strategy based on Cre-Lox recombination of the SV40 T viral antigen.[16] No one has yet achieved the goal of generating a safe and fully-functional yet clonal, immortalized, or genetically-engineered human cell that can be substituted for primary hepatocytes. Besides providing a sufficient number of cells for BALs, proliferating cells would have the advantage of being able to be genetically engineered to overexpress metabolically significant genes (e.g., glutamine synthetase, cytochrome P450s, etc.). Currently, the only way to permanently integrate transgenes into the cellular genome is to infect replicating cells with retroviruses.

Recent data suggest that there are stem cells in the liver that can differentiate into fully mature hepatocytes.[17] Furthermore, studies in rats, mice, and humans have shown that bone marrow is a major extrahepatic source of stem cells that may contribute to liver regeneration after hepatic injury. Pending more research on the mechanisms of stem cell differentiation into hepatocytes, stem cells remain a potentially significant source of liver cells.

In BAL devices tested so far in the clinic, the only human cell used has been the hepatoma C3A line,[15,18] which originated from a tumor and may have signficantly lower detoxification activities than primary hepatocytes. Most current BAL concepts are based on the use of pig hepatocytes. These present no risk of transmitting malignancies to the patient, but pose the risks of hyperacute rejection,[5] transmission of zoonoses,[6] and potential mismatch with human liver functions. Specialized breeding of transgenic animals could address the first two concerns. Very little is known about the metabolic compatibility of liver functions across species. Xenotransplantation studies may help provide useful information in that respect, once there is reliable success in overcoming hyperacute rejection and early death after clinical xenotransplantation.

Development and Optimization of Hepatocyte and Liver Preservation Techniques

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.[19,20]

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.[21] 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.

ENGINEERING A BIOARTIFICIAL LIVER FUNCTIONAL UNIT

Hepatocyte Function and the Environment

The basic functional unit in the liver is the hepatic lobule (Fig. 2), where hepatocytes are organized into a radial network of plates that are one cell thick. On each side of the plate is a thin layer of extracellular matrix (ECM) material that is covered by vascular endothelial cells. Other cell types, such as fat-storing cells and macrophages are dispersed throughout the lobule. Epithelial cells form bile ductules that collect the bile secreted by the hepatocytes. In the lobule, blood flows from the periportal outer region toward the central hepatic vein through specialized porous capillaries called sinusoids. Hepatocytes in the periportal, intermediate or centrilobular, and perivenous zones exhibit different morphological and functional characteristics. For example, urea synthesis is a process with high capacity to metabolize ammonia and
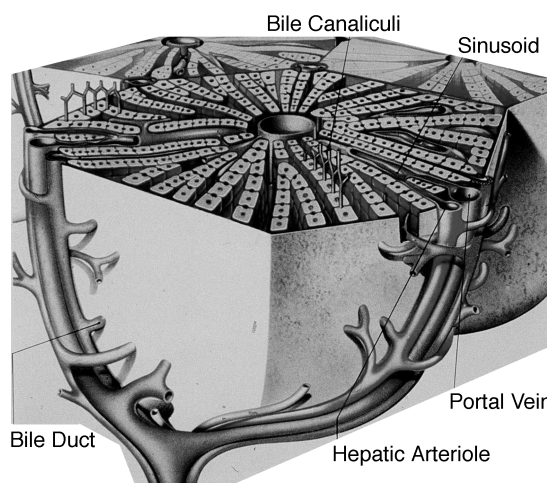

Fig. 2 Structure of the liver lobule. (Reproduced from Ref. [11] by permission of the publisher.)

low affinity for the substrate, which occurs in the periportal and intermediate zones. In the perivenous zone, ammonia is removed by glutamine synthesis, a high-affinity but low-capacity process that removes traces of ammonia that cannot be metabolized by the urea cycle. Replicating the functional heterogeneity of hepatocytes in the lobule may be important to maximize the performance of the BAL.

The maintenance of functional heterogeneity in the liver is dependent on several factors, including gradients of hormones, substrates, oxygen, and ECM composition.[22] In one study where hepatocytes were chronically exposed to increasing oxygen tensions within the physiological range of about 5 mm Hg (perivenous) to 85 mm Hg (periportal), urea synthesis increased about tenfold, whereas cytochrome P450-dependent detoxifying activity decreased slightly and albumin secretion was unchanged.[23] These data suggest that by creating environmental conditions that emulate certain parts of the liver sinusoid, it is possible to modulate hepatocyte metabolism in a way that is consistent with in-vivo behavior, and to purposely upregulate or downregulate specific liver metabolic functions.

Biomaterials in Liver Tissue Engineering

Materials used in tissue engineering

Most of the natural ECM materials used for liver cell culture were obtained via solubilization of tissues by chemical processing. These protein solutions can often be reconstituted into three-dimensional gels of any shape or form, and retain many chemical features of the ECM

proteins, including bound growth factors, found in vivo. Commonly used reconstituted matrices include type I collagen, which is isolated from rat tail or bovine skin by mild acid treatment. The acid solution of collagen can be induced to form a gel upon restoring a physiological pH of 7.4, which causes the polymerization of collagen molecules into a large network of fibrils. The extent of cross-linking in this collagen is very low in comparison with that of the native tissue, but chemical cross-linking can be induced by either glutaraldehyde or dehydrothermal (vacuum and $\sim 100°C$) treatment.

Naturally derived matrices provide good substrates for cell adhesion because cells express the adhesion receptors that specifically recognize and bind to ECM molecules that make up these matrices. Nevertheless, there have been considerable advances in the development of synthetic biocompatible polymers, which theoretically have an unparalleled range of physical and chemical properties. In practice, however, most studies have used relatively few synthetic materials, in part due to a reluctance to expend time and money to secure regulatory approval for clinical use of untested biomaterials.

Optimization of surface chemistry

Cells do not usually directly attach to artificial substrates, but rather to ECM proteins that are physically adsorbed (i.e., by virtue of hydrophobic and electrostatic interactions) or chemically (i.e., via covalent bonds) attached to the surface. Surface treatment, such as with ionized gas, is often used to favor protein adsorption. This process only modifies the surface of the material, and thus minimally affects its bulk mechanical properties. Surfaces have been coated with positively charged materials such as poly-L-lysine to promote cell adhesion by attractive electrostatic interactions with the cells. This is because the latter usually display a negative surface charge due to the presence of negative sialic acid residues on their surface glycocalyx.

Because physisorption is notoriously nonselective, covalent modification of substrates or chemisorption is used if necessary to provide more control over the type, density, and distribution of adhesive protein on the surface of the material. The first step involves using a reactive chemical that bonds to the surface and has a free functional group that easily reacts with free thiol, hydroxyl, carboxyl, or amine groups on proteins. This step often requires harsh chemical conditions, whereas the second step (which involves conjugation of the protein) can be accomplished under physiological conditions. This approach is also suitable to graft small adhesive peptides (e.g., arginine-glycine-aspartic acid (RGD)-peptide) that otherwise would not stably bind to surfaces by physisorption.

Various ECM proteins, such as collagen and laminin, regulate the activities of transcription factors that control the expression of liver-specific genes such as albumin. It has been hypothesized that these factors bind ECM-responsive elements. It is important to realize that no matter what type of surface is used to seed the cells, over a period of several days of culture, cells will have secreted significant quantities of their own ECM onto the substrate, and the initial surface properties of the material will be dramatically altered. For example, cultured hepatocytes continually synthesize collagen, fibronectin, and laminin,[24] and this secretion is important for the maintenance of long-term liver-specific protein secretion in hepatocytes cultured in collagen gels.

More chemically complex ECM, such as matrix extracted from Engelbroth-Holmes sarcoma (EHS) tumors grown in mice, whose composition more closely resembles basal lamina (it consists predominantly of type IV collagen, laminin, and heparan sulfate proteoglycan), induce the expression of liver-specific functions and phenotypic features typical of hepatocytes in vivo.[25,26] There is evidence that the mechanochemical properties of such surfaces, whether they favor cell spreading, rounding, or aggregation, impact the responsiveness of the hepatocytes to growth and other trophic factors.[27]

Extracellular matrix and cell polarity

Extracellular matrix geometry (or topology) affects hepatocyte morphology and function. The effect of matrix topology was investigated in a controlled fashion using a culture technique whereby cells cultured on a single surface were overlaid with a second layer of ECM, thereby creating a sandwich configuration around the cells. In the case of hepatocytes, the overlay establishes an ECM configuration that resembles that found in the liver (i.e., where hepatocytes are generally bounded by ECM at each of their opposite basolateral membrane domains). The result is that hepatocytes remain as a monolayer, but exhibit a dramatic increase in the expression of liver-specific functions compared to cultures on a single ECM-coated surface.[24]

Rat hepatocytes cultured in the collagen sandwich, when compared to a single collagen gel substrate, have a dramatically altered organization and expression of cytoskeletal proteins. The collagen sandwich induces the formation of distinct apical and basolateral membrane domains, each expressing specific surface markers[24] (Fig. 3). Cytoskeletal actin filaments are concentrated under the plasma membrane in regions of contact with neighboring cells, forming the sheathing of a functional bile canalicular network. In contrast, hepatocytes cultured on a single gel exhibit actin-containing stress fibers on the
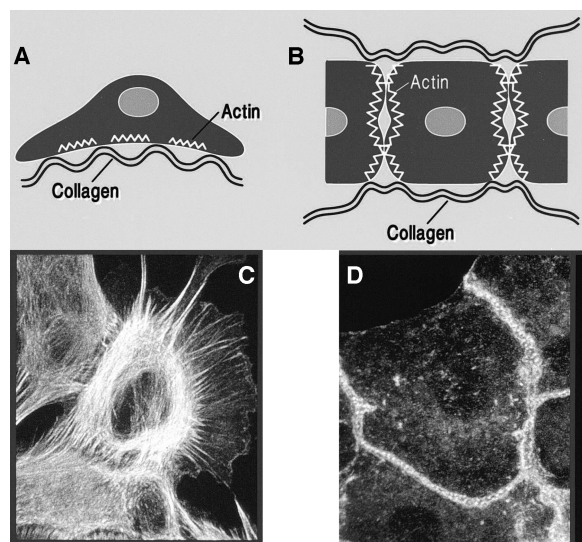
Fig. 3 Morphology and localization of actin fibers in hepatocyte cultures on a single collagen gel (A and C) and in a collagen sandwich (B and D). A and B show the distribution of apical and basolateral membranes in relation to the localization of actin. C and D show actual immunofluorescence staining of actin filaments in rat hepatocytes. (C and D reproduced from Ezzell, R.M., et al., Exp Cell Res **1993**, *208*, 442–452, by permission of the publisher.)

ventral surface in contact with the substrate and no bile canaliculi, a pattern more typical of fibroblasts.

Controlling cell–cell interactions

In general, when cells are seeded on a substrate, they initially form a monolayer that is held together by both cell-substrate and cell–cell adhesions. If the substrate is only moderately adhesive, cell–cell adhesions may be stronger and the cells may spontaneously reorganize into compact aggregates that interact minimally with the surface. When hepatocytes are induced to form aggregates, they maintain viability and stable expression of liver-specific functions for several weeks. Furthermore, recent data show that hepatocytes in spheroids form functional bile canaliculi, which indicates that the cells exhibit distinct apical and basolateral membrane domains.[28] Hepatocyte spheroids have been used as the basis for several BALs.[29]

Approximately 20 years ago, it was discovered that hepatocytes could be cultured on feeder (or supportive) cells to maintain their viability and function.[30] More recent studies showed that nonhepatic cells, even from other species, may 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 coculture systems include the potential variability in the cell line used and the additional work needed to propagate that cell line and attend 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 cocultures. 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 much 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 to ensure that each hepatocyte is near a feeder cell while minimizing the number of feeder cells.[31] 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 ECM or other cell attachment factors onto surfaces have been developed.[32] Photolithography involves spin-coating a surface (typically silicon or glass) with a layer approximately 1 μm thick of photoresist (a photo sensitive chemical resistant to acid), exposing the coated material to ultraviolet light through a mask that contains the pattern of interest, and treating the surface with a developer solution that dissolves only the exposed regions of photoresist (Fig. 4). This process leaves photoresist in previously unexposed areas of the substrate. The exposed areas of substrate can be chemically modified for attaching proteins, etc., or can be treated with hydrofluoric acid to etch the material. The etching time controls the depth of the channels created. Subsequently, the leftover photoresist is removed using an appropriate solvent, leaving a surface patterned with different molecules or grooves. A disadvantage of this method is that it uses chemicals that are toxic to cells, and generally harsh conditions that may denature proteins.

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 μm 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.
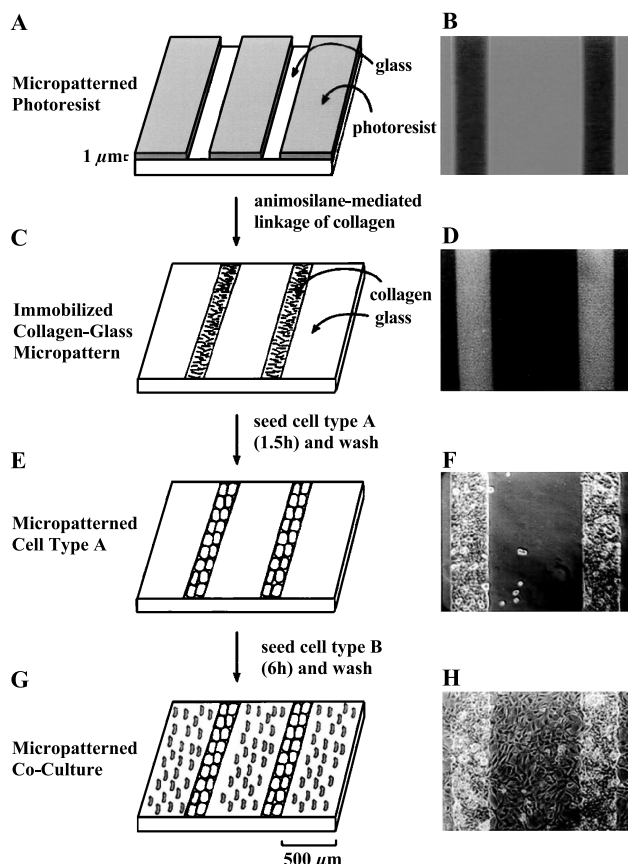
Fig. 4 Photolithographic technique for patterning cells on a glass surface. (Reproduced from Ref. [28] by permission of the publisher.)

Soft lithography methods can be used on virtually any type of surface, including curved surfaces, owing to the flexibility of PDMS. In using these approaches, it is important that the base material be resistant to physisorption, or the selectivity of the adhesive groups may be significantly reduced in vivo. A successful approach to prevent adhesion to the base material is via covalent attachment of antiadhesive factors on the remaining functional groups.

Porous three-dimensional matrices

Porous matrices with pore sizes in the range of 30–300 μm have been used to culture hepatocytes, and have been prepared by salt-leaching or freeze-drying techniques. The first method involves adding water-soluble crystals (e.g., NaCl) of size range similar to the desired pores in the melted base polymer material. After solidification of the polymer, the salt crystals in the resulting solid are dissolved by exposure to aqueous solutions, leaving a pore in the place of every crystal. An alternative approach is to use supercritical carbon dioxide to create pores by the induction of microbubble formation within the polymer. The freeze-drying technique is based on the general principle that when freezing a solution, the solvent forms pure solid crystals while all solute materials are concentrated in the remaining unfrozen fraction. During the subsequent drying process, the solid crystals evaporate and leave pores. The morphology of the solid crystals is dependent on the physicochemical properties of the solution, the temperature gradient at the liquid–solid interface, and the velocity of that interface. Furthermore, the solid crystals tend to orient in the direction of the temperature gradient, so that the direction of the pores can be controlled as well. For practical applications, however, it is more typical to freeze solutions containing biomaterials in a bulk fashion. It is noteworthy that the rate of freezing and the temperature gradient are difficult to control and to maintain constant throughout the freezing process. Thus, porous materials made by this technique typically exhibit nonuniform pore sizes as one moves from the surface to the center.

Per unit volume, such porous materials have a large surface area; at least theoretically they can support the attachment of a large number of cells. This is in practice not the case because of the lack of efficient oxygen transport in porous matrices that lack a functional vascular system or other means to provide convective (i.e., flow-driven) delivery of nutrients. Pore size may affect whether the cells spread onto the substrate or invade the pores, in which case they tend to form three-dimensional aggregates. Such aggregates have been shown to express high levels of liver-specific function, presumably due to the formation of specialized cell–cell contacts among the hepatocytes.[33]

Soluble Factors

Hepatocyte culture media

Certain hormonal additives have been found to be essential for the maintenance of long-term protein secretion and viability in cultured hepatocytes. For example, supplementation with corticosteroids such as hydrocortisone and dexamethasone is used to promote the expression of liver-specific functions in various hepatocyte culture configurations. Serum-free cultures do not benefit from the contribution of hormones and other metabolites found in the serum, and have more requirements in this respect. For example, serum-free cultures of hepatocytes require exogenous insulin and proline.[24] Insulin is an anabolic hormone, and proline is necessary for endogenous collagen synthesis. It is noteworthy that the expression of liver-specific genes in cultured liver slices is only slightly better in hormonally supplemented medium as opposed to basal medium. Further refinements in the composition of the ECM may thus relax some of the

requirements for hormonal and other supplements in the culture medium.

Effect of plasma and blood

Rat hepatocytes that are seeded and maintained in standard hepatocyte culture medium and then exposed to either rat or human plasma become severely fatty within 24 hours, with a concomitant reduction in liver-specific functions. Plasma thus appears to be a rather inhospitable environment for hepatocytes, although it is clear that hepatocytes must be made to tolerate plasma 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 those found in standard hepatocyte culture medium) eliminates intracellular lipid accumulation and restores albumin and urea synthesis as well as P450-dependent detoxification.[34,35] However, direct supplementation of plasma, especially with respect to the high levels of hormones used, is high in cost and potential health risk to the patient.

Hepatocytes are cultured in an artificial medium for several days prior to their use in a BAL, where they will contact plasma or blood from the patient. Culture media containing supraphysiological levels of hormones—and more specifically, insulin (used at about 10^4 times physiological concentration in standard hepatocyte culture medium)—predispose the hepatocytes to an abnormal response when subsequently exposed to plasma. In a recent study in our laboratory, we found that preconditioning hepatocytes in low insulin-containing medium reduced intracellular lipid accumulation during subsequent plasma exposure.[36] Direct amino acid supplementation of plasma increased both urea and albumin secretion rates by the hepatocytes. We thus concluded that a combination of both preconditioning and plasma supplementation can be used to upregulate liver-specific functions of hepatocytes during plasma exposure.

BIOREACTORS

Transport Issues in Scale-up of Hepatocyte Cultures

In normal liver, no hepatocyte is farther than a few micrometers from circulating blood; thus, transport by diffusion has to occur only 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 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, makes oxygen transport the most constraining parameter in the design of BAL devices.

Oxygen transport and uptake of hepatocytes have been extensively studied in the sandwich culture configuration in order to obtain the essential oxygen uptake parameters needed for the design of bioreactor configurations.[37] The maximum oxygen uptake rate of cultured rat hepatocytes was measured to be about 13.5 pmol/s/µg DNA, which is fairly stable after the first day in culture and continuing for up to 2 weeks. Interestingly, the oxygen uptake was about twice on the first day after cell seeding, presumably because of the increased energy required for cell attachment and spreading. Perhaps this should be taken into account when seeding hepatocytes into a BAL. Oxygen uptake was not sensitive to oxygen tension in the vicinity of the hepatocytes up to a lower limit of about 0.5 mm Hg. Below this, oxygen uptake decreased, suggesting that it becomes a limiting substrate for intracellular hepatocyte metabolism. Because oxygen is essential for hepatic adenosine triphosphate (ATP) synthesis, a reasonable design criterion would specify that oxygen tension remain above approximately 0.5 mm Hg.

Based on these parameters, it is possible to estimate oxygen concentration profiles in various bioreactor configurations based on a simple diffusion-reaction model that assumes that the process follows Michaelis–Menten kinetics. One can generally 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.[38] Calculations of 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 300–500 µm. At cell densities of 10^8 cells/cm^3, which is similar to those found in normal liver, that thickness is only 100–200 µm.

Hollow-Fiber Systems

The hollow-fiber system has been the most widely used type of bioreactor in BAL development.[39,40] 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 that does not contain the cells is generally perfused with culture medium or with the patient's plasma or blood. The fiber walls may provide the attaching surface for the cells or act as a barrier against the immune system of the host. Microcarriers have also been used as a means to establish an attachment surface for anchorage-dependent cells introduced in the shell side of hollow-fiber devices. Many studies have been conducted to determine optimal fiber

dimensions, spacing, and reactor length based on oxygen transport considerations.[41]

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 diameter. 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 be perfused with hormonal supplements, etc., to enhance the cells' viability and function 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, additional fibers that carry oxygen straight into the device have been used.[29,42]

Parallel Plate Systems

An alternative bioreactor configuration is based on a flat surface geometry[37] where it is easier to control internal flow distribution and ensure that all cells are adequately perfused. The main drawback to this configuration is that it is difficult to build a system that contains sufficient cell concentration (Fig. 5). For example, a 1 mm channel height would result in a 10-liter reactor to support 20×10^9 hepatocytes cultured on a surface 10 m^2 in area. For a liver failure patient who is probably hemodynamically unstable, it is generally accepted that the priming volume of the system not exceed 1 liter.

The volume of the device in the flat-plate geometry can be decreased by reducing the channel height (Fig. 5). However, this forces the fluid to move through a smaller gap, which rapidly increases the drag force (shear stress) imparted by the flow to the cells. Recent data suggest that hepatocyte function decreases significantly at shear stresses >5 dy/cm^2.[37] To reduce the deleterious effects of high shear, it may be possible to use grooved surfaces, wherein cells lodge and are less exposed to shear stress, allowing for faster flow without causing cell damage.

In an attempt to provide adequate oxygenation to cells and to protect them from shear in perfused bioreactors, gas-permeable membranes and membranes separating cells from plasma have been incorporated into the flat-plate geometry. A flat-plate microchannel bioreactor wherein cells directly contact the circulating medium has been developed.[37] 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 wherein a nonpermeable glass surface is substituted to the membrane, internal membrane oxygenation removed the oxygen limitations that occur at low volumetric flow rates.

Monitoring the Performance of Bioartificial Livers

The performance of BALs is often assessed based on clinical parameters such as survival, grade of encephalopathy, and blood and urine chemistry values. Because these parameters can vary from one patient to another depending on the severity of the disease, it is worthwhile to assess BAL function through other methods. Further insight into the function of the BAL can be obtained by measuring the change in metabolite levels across the BAL, including oxygen, CO_2, ammonia, urea, amino acids, and ketone bodies.

An enhanced perspective of metabolism and cellular function can be obtained with a mathematical modeling framework that considers the stoichiometric constraints of the intracellular reaction network of the cells in the BAL.
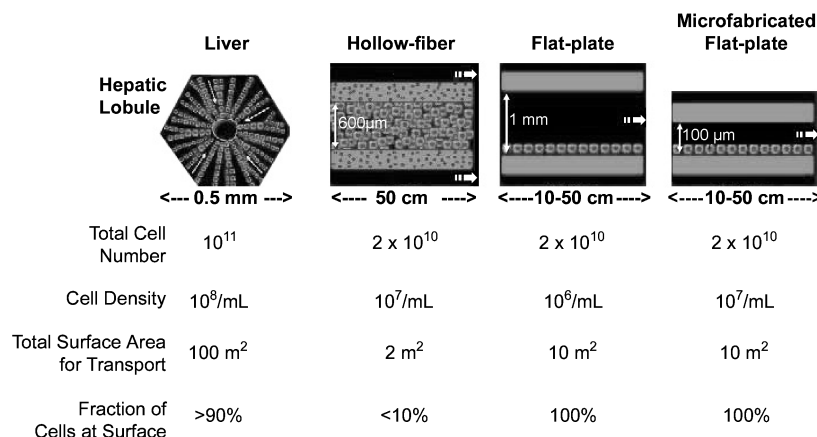
	Liver	Hollow-fiber	Flat-plate	Microfabricated Flat-plate
Hepatic Lobule	<--- 0.5 mm --->	600μm <---- 50 cm ---->	1 mm <----10-50 cm---->	100 μm <----10-50 cm---->
Total Cell Number	10^{11}	2×10^{10}	2×10^{10}	2×10^{10}
Cell Density	10^8/mL	10^7/mL	10^6/mL	10^7/mL
Total Surface Area for Transport	100 m^2	2 m^2	10 m^2	10 m^2
Fraction of Cells at Surface	>90%	<10%	100%	100%

Fig. 5 Comparison among popular hepatocyte bioreactor configurations.

One such methodology that is especially useful for the analysis of the metabolic function of organs and tissues is metabolic flux analysis. Metabolic flux analysis refers to the calculation of intracellular rates of metabolic reactions using steady-state metabolite balance models applied to measured rates of uptake and release of extracellular metabolites. This approach has been extensively used to study and improve strains of microorganisms (bacteria and yeasts) of significance in biotechnology, and has more recently been applied to mammalian cell systems, including perfused liver.[43] Metabolic flux analysis, once validated for the particular case under study, is potentially very useful as it is noninvasive and cost effective.

CONCLUSION

The extracorporeal bioartificial liver is a promising technology for the treatment of liver failure, but significant technical challenges remain before systems with sufficient processing capacity and of manageable size can be developed. Most efforts to date have focused on device design and construction, and more recently on the development of methods to generate a continuous supply of human hepatocyte cell lines. New designs are not yet able to stably sustain the large cell populations needed for therapeutic purposes, and concerns about the safety of transformed cell lines remain. On the other hand, there have been fewer efforts to improve the specific functional capacity of the hepatocytes used in bioartificial livers. Increasing the efficiency of the cellular component of bioartificial livers would greatly facilitate their design. The functional capacity of cells used in bioartificial livers may be improved on at least three different levels: 1) by altering the internal machinery of individual cells to upregulate critical functions; 2) by judiciously controlling the spatial distribution of multiple cell populations with different specializations to emulate the organization of the liver acinus; and 3) by optimizing the overall treatment protocol, including the interval, duration, and number of treatment sessions.

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ARTICLES OF FURTHER INTEREST

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