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Review

Liver Cell Culture Devices

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In the last 15 years many different liver cell culture devices, consisting of functional liver cells and artificial materials, have been developed. They have been devised for numerous different applications, such as temporary organ replacement (a bridge to liver transplantation or native liver regeneration) and as in vitro screening systems in the early stages of the drug development process, like assessing hepatotoxicity, hepatic drug metabolism, and induction/inhibition studies. Relevant literature is summarized about artificial human liver cell culture systems by scrutinizing PubMed from 2003 to 2009. Existing devices are divided in 2D configurations (e.g., static monolayer, sandwich, perfused cells, and flat plate) and 3D configurations (e.g., liver slices, spheroids, and different types of bioreactors). The essential features of an ideal liver cell culture system are discussed: different types of scaffolds, oxygenation systems, extracellular matrixes (natural and artificial), cocultures with nonparenchymal cells, and the role of shear stress problems. Finally, miniaturization and high-throughput systems are discussed. All these factors contribute in their own way to the viability and functionality of liver cells in culture. Depending on the aim for which they are designed, several good systems are available for predicting hepatotoxicity and hepatic metabolism within the general population. To predict hepatotoxicity in individual cases genomic analysis might be essential as well.

Key words: Cell culture devices; Human liver cells; Drug delivery; Bioreactor

INTRODUCTION

In the last 15 years many different liver cell culture devices, consisting of functional liver cells and artificial materials, have been developed. They have been devised for numerous different applications, such as temporary organ replacement (a bridge to liver transplantation or native liver regeneration) and as in vitro screening systems in the early stages of the drug development process, like assessing hepatotoxicity, hepatic drug metabolism and induction/inhibition studies.

Recently, an increased number of approved drugs and new chemical entities (NCE) have been withdrawn from the market, because of low pharmacokinetics/pharmacodynamics profiles, or serious and unexpected adverse effects during postmarketing surveillance phase, leading to high costs and unacceptable prolonged times for drug development (29,43,97).

Because the liver is the key player in drug metabolism, the challenge still exists to develop an in vitro liver cell system able to effectively predict, in a species-specific manner, the liver toxicity, the biotransformation reactions, and the potential for interactions of drugs and NCEs in the preclinical stage of drug discovery and development.

Furthermore, the development of an in vitro screening system, based on living human liver cells, might be an alternative to animal experimentation. It bypasses the lower predictive value of animal models related to significant interspecies differences and bioethical considerations, reducing animal use for research purposes.

Multiple efforts have been made within the scientific community in order to find a cell-based system able to assess human hepatotoxicity of NCEs and new drugs as well to study in vitro hepatic metabolism.

This review summarizes as much as possible the experimental data regarding two-dimensional (2D) and three-dimensional (3D) in vitro screening systems based

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on (mainly human) hepatocytes intended for evaluating liver cell functionality, toxicity, and intermediary metabolism. PubMed was screened between 2003 and 2009 using the terms "hepatocyte in vitro system" and "hepatocyte and toxicology screening." Furthermore, we tried to identify the landmarks of the optimal in vitro system (44).

In general, in vitro systems of human liver cell cultures should be optimized, because they are the only acceptable alternative to study hepatotoxicity and drug metabolism preclinically. In vitro studies with animal liver cells, perfused whole livers, or even in vivo studies in intact animals are less informative for the clinical application.

Various in vitro systems have been studied, such as primary human liver cells, human liver cell lines, subcellular systems (microsomes and mitochondria), and a variety of recombinant systems (29,44,97). Each of the aforementioned systems shows advantages and disadvantages to be considered when choosing a system that best simulates the in vivo situation.

However, in vitro systems, like isolated liver cells, subcellular systems, and cell lines, differ from the complex spectrum of metabolism and gene expression of cells in vivo, and therefore have to be considered as second best. Subcellular fractions, like microsomes, inadequately represent the diversity of hepatic functions and can only be used for very specialized functions (45).

For these reasons we restricted ourselves in this review to in vitro models of intact (mainly human) liver cells in 2D and 3D configurations (15,60).

Isolation procedures for hepatocytes have been optimized by now and make it possible to obtain high amounts of viable cells (24,30,40). Freshly isolated human hepatocytes are able to perform the full range of hepatic functions (i.e., biotransformation, synthesis, and detoxification).

TWO-DIMENSIONAL (2D) CONFIGURATIONS

Cell Suspensions

Freshly isolated or cryopreserved hepatocytes in suspension represent a readily available and easy-to-handle in vitro system that can be used to characterize the metabolism of test substances. Additionally, they are able to ensure a good transfer between culture medium and hepatocytes. A limitation of suspensions is the length of the incubation period, often limited to a few hours: this incubation period is sufficient to determine the metabolic stability and to allow identification of the main metabolites of a test substance, but may be too short to allow the generation of phase II metabolites, because their contribution is less than 3% of total metabolism (37).

Static Monolayer Cultures

Static monolayer culture systems are characterized by an unstirred medium layer overlying cells that are attached to a gas-impermeable membrane and are exposed to changes in nutrient concentrations and catabolite accumulation over time. Periodic refreshment of culture medium removes accumulated catabolites and renews nutrients. Oxygen is applied by culture gas (mostly carbogen) in an incubator. Sterile conditions prevent bacterial and fungal infection. When cultured on plastic or collagen-coated dishes, hepatocytes give arise to a system that is simple to be used, able to preserve cell–cell interactions, and to show liver specific function for up to 24 h. Under standard adherent culture conditions, they can provide a wide range of applications: short-term hepatotoxicity, cytochrome P450 induction and inhibition, drug interaction, pharmacokinetics, and pharmacodynamics. However, there is not a commonly accepted protocol for culturing human hepatocytes; thus, different culture media [William's E medium (WE), Dulbecco's modified Eagle's media (DMEM), Hanks' balanced salt solution (HBSS), hepatocyte maintenance media (HMM), Leibovitz's L15, modified Chee's medium, etc.] and a variety of supplements (fetal serum, amino acids, hormones, growth factors, peptides, cytokines, etc.) are used by researchers.

There are also multiple types of cell scaffolds to which the hepatocytes are attached (41). Plastic plates are used as such or covered by a single layer of extracellular matrix (collagen, fibronectin, and laminin), or a complex extracellular matrix, like Matrigel® (an extract derived from the basement membrane of the Engelbroth-Holm-Sworm mouse sarcoma), whereas an extracellular matrix overlay or sandwich cultures are used by other researchers. Culturing hepatocytes on dishes coated with extracellular matrix proteins has been found to retain cell morphology and metabolic functions better than cells cultured on uncoated plastic (29).

Sandwich Configurations

Using collagen type I–collagen type I or collagen type I–Matrigel® layers, liver cells best maintain their cell morphology and architecture. Albumin secretion is maintained for several weeks (71) as well as induction capacity for both CYP1A2 and CYP3A4 (61). Collagen–Matrigel® sandwiches have shown additional advantages with respect to collagen–collagen matrices and monolayer collagen, such as expression of epidermal growth factor receptor (EGF-R), connexin 32, a gap junction protein (97), as well as markedly stronger induction of CYP 2B1/2 by phenobarbital (29).

However, Matrigel® has some disadvantages; it is more expensive than collagen type I and has variations

in composition from batch to batch. In addition it is xenogenic for human cell culture use.

In static monolayer cultures the expression and activity of many phase I drug-metabolizing enzymes is significantly downregulated. Static cultures reduce cell signaling, playing an important role in maintaining stable liver-specific functions, and create non-steady state conditions by reduced substrate concentrations and accumulation of waste products (29). Static culture systems are simple to use, but they lack the complexity of in vivo liver tissue.

Perfused Cultures

2D perfused cultures are based on hepatocytes cultured on collagen-coated slides perfused with medium in a constant atmosphere of oxygen–nitrogen–CO_2. Such systems, in contrast to static culture models, have been shown to better sustain liver cell functions for at least 2 weeks and guarantee a remarkable sensitivity to enzyme induction (37).

The application of direct fluid flow parallel to the cell surface is not physiological, because hepatocytes in vivo are not in direct contact with flowing blood. However, the set up of a perfused culture system with an interstitial-like flow can allow the passage of essential nutrients and catabolites, better simulating the architecture and the supply/removal of substances by the bloodstream as it is in vivo.

Thus, an in vitro system that can combine in a single design each aspect (maintenance of heterotypic interactions, preservation of aspects of liver architecture, and modulation of fluid flow stresses) might be a milestone in the development of a model that resembles the full spectrum of physiological hepatocyte functions.

Flat-Plate Cultures

Flat-plate culture systems have several positive features. The cells are able to adhere to the plates in order to create a homogenous microenvironment, as well as the culture medium/plasma can adequately come into contact with seeded cells, which allows sufficient mass transfer. In addition, such a system can easily be scaled up and allow high-throughput screening or studying of zonation-dependent phenomena involving drug metabolism and toxicity (4).

One major disadvantage of flat-plate culture systems is that the cells are exposed to high-shear stress. Consequently, they may detach from their scaffold and quickly lose their viability and function because they are anchor-dependent cells.

In order to solve this problem, Park designed a flat-plate culture system with microfabricated grooves. In the bioreactor, nanometer materials were introduced by electrospinning the chitosan (a polysaccharide polymer produced by alkali deacetylation of chitin) nanofibers into the plates (67). Through visual inspection, hepatocytes cultured on chitosan nanofiber plates exhibited a superior adhesion ability compared with plates without nanofibers. Furthermore, the scanning electron microscopy (SEM) images showed pseudopod formations, which further facilitated cells to tightly adhere to the nanofiber surface. The novel design of this culture system reduced shear stress forces by as much 30 times and ensured stable rates of albumin and urea synthesis of hepatocytes cocultured with 3T3-J2 fibroblasts over 5 days of perfusion. Thus, providing new promising insights in the control of shear stress on liver cells and in the scaling-up of the system for the development of a functional bioartificial liver (77).

THREE-DIMENSIONAL (3D) CONFIGURATIONS

Liver Slices

Liver slices are able to preserve the integrity of cell–cell and cell–extracellular matrix (ECM) interactions. They contain nonparenchymal cells (that play a very important role in mediating toxicity) and are well-accepted systems, being available since 1980. In addition to general metabolic studies, zone-specific toxicity (29) can be assessed. However, availability of human liver slices is extremely restricted and hepatic functions do not remain stable, because of the limited diffusion of oxygen and nutrients from the medium stream to the cells (appropriate section thickness = 150–250 μM) (29,45).

Spheroids

When hepatocytes are weakly adherent or nonadherent to a substrate they aggregate in suspension to form spheroids (29,102). Two characteristic formations of spheroidal aggregates are important in the maintenance of liver differentiated functions; the establishment of 3D cell–cell contacts and the secretion of extracellular matrix proteins within the spheroid.

Spheroidal cultures have limited application because of the presence of hypoxic/necrotic cells in the center of the spheroid due to low oxygen diffusion or accumulated bile acids. However, Powers et al. (84) showed that a bioreactor seeded with hepatocyte spheroids maintains long-term architecture and viability, whereas structures formed from seeding single cells were transient. These observations indicate that hepatocytes in a 3D configuration maintain the longest viability.

The Mayo Spheroid Reservoir Bioartificial Liver system is composed of two components: the multifunctional Spheroid Reservoir and the Multi-Shelf Rocker. The Spheroid Reservoir provides an environment to support the viability and functionality of 200–400 g of hepatocyte spheroids at very high cell densities; the Multi-

Shelf Rocker fulfills the production requirement by allowing the culturing of up to 6 L of hepatocyte suspension in a conventional laboratory incubator. Such a system is designed to provide life-sustaining liver-like function as a bioartificial liver to patients with acute liver failure (69).

Bioreactors

In general four types of bioreactors used for liver cell culture are distinguished: 1) hollow fiber, 2) flat plate and monolayer, 3) perfused beds and scaffolds, and 4) encapsulation and suspension (Table 1).

Bioreactors types 1, 3, and 4 allow the monoculture or coculture of hepatocytes under tissue-specific mechanical forces (pressure, shear stress, flow) in a 3D framework (39,63,66). Some of these bioreactors have been used as bioartificial livers (BALs), charged with various types of liver cells, to bridge acute liver failure patients to transplantation (8,10,11,21,35,52,53,74,83, 88,90,91,95,99,100,104–107,111). The actual challenge is to use the cell-based bioreactors as in vitro screening systems for drug toxicity and metabolism evaluation.

Hollow Fiber (HF). In most semipermeable HF bioreactors the external site of the HF is used for cell attachment, while perfusion medium is circulated through the internal lumen. These devices avoid shear stress to the cells, but have the disadvantage of mass transfer restrictions with regard to import to the cells of nutrients and oxygen and export from the cells of waste products, carbogen, and metabolic products.

Sullivan et al. (98) show in the O_2 transport model that the proper O_2 spectrum cannot be fully achieved with the current HF bioreactors. The fiber spacing must be chosen carefully to ensure that the extracapillary space is large enough for the maintenance of a functional number of hepatocytes, while at the same time the extracapillary space surrounding each fiber must be small enough to provide sufficient oxygenation to the attached hepatocytes. For this reason the inlet oxygen partial pressure ($pO_{2\ IN}$) needs to be raised above the physiological O_2 concentration.

The supply of oxygen can greatly be improved by an extra set of oxygen-permeable fibers interwoven with the cell-containing fibers, as is done in the bioreactor from Charite (39).

Other HF bioreactors use the intraluminal space for cell attachment in collagen gel and medium is perfused via the same lumen (77): this type of bioreactor has less restrictions of mass transfer, but again oxygenation is not optimal.

For the most part, HF bioreactors with and without cells have represented the most common applied technology for use in kidney dialysis and in clinical applications in acute liver failure patients. Currently no cell bioreactor is commercially available for routine clinical application, while several systems providing the HF technology have undergone clinical trials (11). The most widely tested device has been the HepatAssist from Circe Biomedical (currently Arbios), integrating $5–7 \times 10^9$ cryopreserved porcine hepatocytes in collagen-coated microcarriers and a charcoal column for removal of absorbants (13). It has been tested in the largest controlled clinical trial of a BAL device with 171 patients; the primary end point, 30-day survival, was achieved in 71% of patients in the BAL treatment group, but was not significantly different from the 62% survival in the control group (18).

Other examples of bioartificial livers used in clinical trials are Vital Therapies extracorporeal liver assist device (ELAD), consisting of a dual pump dialysis system and hollow fiber cartridges containing a hepatoma-derived cell line, C3A. A pilot-controlled trial was performed in 24 patients: improvements were seen in mental status, renal function, and hemodynamic stability, but not on survival (25).

The MELS BAL system is a more complex bioreactor based on interwoven HF membranes, creating a 3D framework over which hepatocyte aggregates are distributed. In addition is a dialysis module present in the

Table 1. Characteristics of Different BAL Bioreactor Designs [Modified From Park and Lee (81)]

	Hollow Fiber	Flat Plate and Monolayer	Perfused Beds/Scaffolds	Encapsulation and Suspension
Pros	Attachment surface; Potential for immunoisolation; Well characterized; Cells protected from shear stress	Uniform cell distribution and microenvironment	Ease of scale-up; Promotes 3D architecture; Minimal transport barrier	Potential for immunoisolation; Ease of scale-up; Uniform microenvironment
Cons	2D cell layer; Nonuniform cell distribution; Transport barrier by membranes or gels	Complex scale-up; Potential large dead volume; Cells exposed to shear forces; Low surface area-to-volume ratio	Nonuniform perfusion; Cells exposed to shear forces	Transport barrier due to encapsulation; Degradation of microcapsules over time

system (39,83). The MELS was the only system that had been used in clinical studies with primary porcine hepatocytes as well as primary human hepatocytes derived from discarded donor livers (90–92). In a phase I study with primary human hepatocytes, eight patients were treated with the MELS system; the overall treatment time ranged from 7 to 144 h. No adverse events were observed and in all eight cases neurologic status improved and slight improvement of coagulation was observed during treatment (92).

Many other HF systems have been involved in clinical applications (9,98), such as the TECA-Hybrid Artificial Liver Support System (TECA-HALSS) and the Hybrid-Bioartificial Liver (HBAL), both developed in China (22,110). Others have undergone extensive experimental testing before proceeding to a clinical trial, such as the systems described by De Bartolo et al. (17), Sullivan et al. (98), and Schwartlander et al. (94).

Perfused Beds/Scaffolds. These systems are not hampered by mass transfer problems as they support direct contact between medium and cells without interposition of a semipermeable membrane, but they do suffer from shear stress problems to the cells.

Hoque et al. (51) described their system as 10 scaffolds positioned centrally along the length of the bioreactor constructed from pure glass tubing and held in place on both ends of the glass tube with biomedical-grade silicon rubber. Polyethylene terephthalate is used as the main scaffolding material, incorporated into the polysulfone hollow fiber using the textile braiding technique. The hybrid scaffold provides a highly organized 3D framework with well-defined pores that increases the surface area of the bioreactor for hepatocyte cultures. In addition, it provides high-density hepatocyte immobilization, preventing the cells from being swept away because of shear stress, and allows better perfusion of hepatocytes, with an ample supply of oxygen and nutrients and removal of waste products. Preliminary results show that the scaffold positively influences the functionality (albumin and urea secretion) of hepatocytes in the bioreactor. These results are promising for further clinical development of the system (51). Another interesting scaffold for developing functional culture methods aimed at potential applications in BAL studies for future clinical use, is the system of Kataoka et al. (54). HepG2 cells can actively proliferate and form cell clusters within 3 days and are able to secrete a one to three times greater amount of albumin than secreted in a monolayer culture.

The radial flow bioreactor of Ferrara comprises a woven–nonwoven polyester matrix sandwiched between two precision woven polyester screens. Cells are injected into the 6-mm-thick polyester matrix and are perifused with oxygenated medium in a radial fashion (72,73).

Naruse et al. used a collagen-coated nonwoven polyester matrix in which preformed hepatocyte aggregates were entrapped (75).

The Academic Medical Center (AMC) bioreactor uses a gas plasma-treated nonwoven polyester matrix to which liver cells are attached as microaggregates. Multiple polypropylene oxygen capillaries are layered between the spirally wound matrix, creating space for medium flow and supplying oxygen to the cells at site. Cells have direct contact with the oxygenated medium (31–33). The bioreactor, charged with $10–15 \times 10^9$ porcine hepatocytes derived from Specified Pathogen Germ Free pigs, was used in a phase I clinical trial in 14 patients with acute liver failure (ALF) or primary nonfunction (PNF), for a maximum time of 35 h, showing improvements in hemodynamics, diuresis, and neurological state without adverse effects. In this study patients in a grade III and IV coma waiting for an orthotopic liver transplantation (OLT) were successfully bridged to transplantation or liver regeneration during a waiting period of several days (21,105,106).

Encapsulation and Suspension. Hepatocytes are encapsulated inside semipermeable alginate gel beads and packed into a column. Such columns can be perfused with oxygenated medium; these systems can be easily scaled-up and do not suffer from shear stress. Disadvantages include poor stability of the hepatocyte suspension, mass transfer problems, and degradation of the microcapsules over time (3,27,70).

Furthermore, alginate-entrapped hepatocytes have been shown to express liver-specific functions in a comparable manner to hepatocyte monolayers, but maintain their functionality longer (34,47).

WHICH CRITERIA SHOULD A LIVER CELL-BASED BIOREACTOR MEET?

It is generally accepted that the scaffold, neighboring cells, nutrients, and oxygen transfer highly influence the viability and functions of hepatocytes. These aspects have to be taken into account when setting up the system best able to mimic the hepatic microenvironment and to promote optimal liver functions.

Scaffold, Membranes, and 3D Configurations

The scaffold to which the cells are attached not only provides a surface for cell adhesion, but also has a profound influence on modulating the cell shape and gene expression relevant to cell growth and liver-specific functions.

If interposed as a membrane between the medium and the cell compartment, they are able to modulate the transport of water and soluble nutrients from medium to

cells and products and waste metabolites from cells to medium (112).

In the beginning membranous scaffolds in bioreactors were based on cellulose acetate dialysis membranes with low nominal molecular weight cut-off and low hydraulic permeability. Subsequent approaches used membranes with a high hydraulic permeability, such as highly permeable asymmetric ultrafiltration membranes with molecular weight cut-off equal to or greater than about 100 kDa and microfiltration (plasmapheresis) membranes with maximal pore size of about 0.2 mm.

Another important aspect of membranes is their chemical composition. Many of them consist of hydrophobic polymeric backbones hydrophilized by chemical attachment of hydrophilic groups or by blending hydrophilic polymers (e.g., polysulphone) or by physical treatment (e.g., polypropylene). Only a few bioreactors use membranes made of hydrophilic polymers (cellulose and its derivatives) with a molecular weight cut-off of about 100 kDa (51,105).

An adequate mass transfer is a critical issue in bioreactor design, especially when cells are cultured in 3D multicellular aggregates or spheroids, where mass transfer limitation of oxygen and metabolites may occur in the core of aggregates (101).

Nowadays the development of 3D configurations, in contrast to 2D ones, could be considered a milestone in cell culture research, because it more closely reflects the cellular microenvironment and the in vivo conditions that promote higher metabolic activity of liver cells.

Monolayer designs can be advantageous in terms of straightforward construction, control of mass transfer resistances, and inspection in situ of the cells; in addition, heterotypic interactions can be realized by adding various cell types in multilayers or in pattered surfaces. Contrary to 2D configurations, the structure of a 3D bioreactor makes the control of oxygen and nutrient transport more complex. It is also more complex for in situ observation of cells. Additionally, 3D architecture provides another dimension for mechanical inputs and cell adhesion, dramatically affecting integrin ligation, cell contraction, and associated intracellular signaling (15).

Schug et al. (93) and Sivaraman et al. (97) have shown that the 3D microreactor culture system, based on the distribution of cells into many tiny tissue units uniformly perfused, maintained expression levels closer to in vivo tissue microenvironment and isolated hepatocytes than either Matrigel® or collagen gel sandwich cultures after 20 days of culture.

The concepts of tissue engineering have been applied in order to create highly porous polymeric matrices as 3D scaffolds, which could be further improved by optimizing shape and composition as well as by attaching growth factors and ECMs (112).

Thirty-five years ago coating surfaces existed of collagen. Later encapsulation in 3D matrices (3D hyaluronan based) allowed to appreciate cell morphology, mechanics, and behavior in a 3D tissue-equivalent environment. Currently new substrata are available, such as PureCol (type 1 collagen) and Extracel (a synthetic covalently cross-linked ECM for 3D cell culture). Other approaches to provide a microenvironment for normal function of hepatocytes include encapsulation in alginate microspheres and HF.

An interesting 3D tissue system has been described by Tsang et al. (103): hepatocytes, in coculture with fibroblasts, are incorporated in polyethyleneglycol (PEG) hydrogels with integrins, synthetic peptides, sugars, and matrix molecules, giving arise to a multilayer 3D tissue structure perfused in a continuous-flow bioreactor.

Oxygenation of Liver Cells

Liver cells are highly oxygen dependent for optimal functionality. In vivo this is guaranteed by the highly oxygenated hepatic arterial blood and the partial oxygen content of portal blood, which is also rich in nutrients. Additionally, the hepatic sinusoid is arranged in such a way that O_2 diffusion distances are minimized (36).

Allen and Bathia discussed the wide range of oxygen tensions inside hepatic sinusoids, accounting for the zonal hepatocyte phenotype variation within the liver sinusoids (2,49,50): the entrance of sinusoids (the periportal afferent region) experiences the highest O_2 tensions ($pO_2 = 60–70$ mmHg). This section feeds into the pericentral zone ($pO_2 = 35–60$ mmHg), where periportal functions taper and perivenous functions are first observed. Lastly, the perivenous efferent region, the least O_2-rich section of the sinusoid ($pO_2 = 25–35$ mmHg), is reached.

This is the reason why the provision of a controlled oxygen gradient from 25 to 70 mmHg within a hepatic HF bioreactor is expected to create functional hepatocyte zonation similar to what is observed in vivo. Cocultures of hepatocytes and nonparenchymal cells (4,41), perfused in oxygen gradients, allowed for differential expression of CYP2B and 3A and toxicity of acetaminophen.

Due to the low solubility of O_2, long diffusion path lengths, and high oxygen consumption rates by hepatocytes, O_2 is a limiting nutrient in bioreactor cultures. It is well known that the natural liver has an extensive sinusoidal network that contains diffusion distances for oxygen and substrates less than approximately 100 μm. For this reason, hypoxic regions in a bioreactor may develop when the diffusion distance exceeds 100 μm.

The amount of oxygen actually dissolved in culture medium is insufficient for cell aggregates to survive in bioreactors. Liver cells in the bioreactor should see ei-

ther full blood or plasma. Full blood is problematic because of hemolysis, clotting, and platelet loss. Plasma necessitates an extra oxygen carrier, such as fluorocarbons or locally supplied oxygen by a so-called internal oxygenator: hollow fiber oxygen capillaries interwoven with the cells, as used in the MELS (90), or the BAL matrix (31–33).

Sullivan et al. (98) tried to supplement the circulating media stream of its HF bioreactor with a hemoglobin-based oxygen carrier (bovine RBCs); Sarwat, as Simoni (96) reported, incorporated perfluorocarbon (PFC) synthetic oxygen carriers into encapsulation matrices; Chu et al. (14) added red blood cells (RBCs) in the circulating medium because they exhibited a sigmoidal oxygen dissociation curve similar to that of blood.

Balance Between Flow Rate and Fluid Shear Stress

Cell oxygenation could be partially controlled as well by varying the medium flow rate, but may consequently apply shear stress on the hepatocytes.

There is a strong correlation between flow and oxygen gradients: it has been calculated that for medium flow rates between 0.45 and 2.2 ml/min, the outlet oxygen concentration of the flat-plate bioreactor is between 106 and 144 mmHg, respectively (26,85).

Tilles et al. (101) developed a microchannel flat-plate bioreactor, charged with rat hepatocytes and an internal gas-permeable membrane through which oxygen is supplied. This system demonstrated that shear stresses greater than 5 dyn/cm^2 resulted in significantly decreased albumin and urea synthesis rates over 3 days of perfusion, whereas at shear stresses ranging between 0.01 and 0.33 dyn/cm^2 the synthesis rates remained stable up to 10 days. Ethoxyresorufin-*o*-deethylation (EROD) activity was not adversely affected by a shear stress of 10 dyn/cm^2 during culturing for at least 12 h (101).

Devarapalli et al. (20) performed tests in two different shapes (rectangular and circular) and six sizes of reactors described by Lawrence et al. (58) using computational fluid dynamic tools. In all designs a 10-fold increase in flow rate augmented the shear stress and the pressure drop by 10-fold.

High flow rates mean high fluid shear stress, high oxygen tensions within the bioreactor, and cell injury induced by the formation of reactive oxygen species (28). Cells respond to the level of hydrodynamic stress by remodeling their surrounding extracellular matrix and changing their tissue composition. Furthermore, high flow rates may deteriorate the quality of the regenerated tissue via washout of the de novo synthesized matrix elements prior to complete assembly and may also affect scaffold degradation rates, influencing in turn its structural and mechanical properties. On the other hand, low flow rates limit the oxygen supply, lead to nutrient deficiency, reduce cell viability and survivability, but ensure lower shear stress.

Lawrence et al. (58) have analyzed the effect of in- and outlet configuration on shear stresses in their reactor. They concluded that the circular design decreased nonuniformity in hydrodynamic stress as well as nonuniform nutrient distribution.

Leclerc et al. (59) used silicon materials for the reactor design due to its high permeability, and combined low flow rates and an external oxygen supply system, in order to deliver an adequate amount of oxygen to the cells.

Park et al. (80) described a system represented by a flat-plate bioreactor, without the use of an internal membrane oxygenator. They fabricated microgrooves onto a glass substrate using photolithographic techniques to reduce the negative effects of shear stress on the cultured hepatocytes. The microgrooves reduce the shear stress 30 times at the cell surface for a given medium flow rate and allow high volumetric flow for adequate oxygen delivery.

Another solution was proposed by Hoque et al. (51): a hybrid scaffold providing a highly organized 3D porous framework where cells can spontaneously reorganize into well-distributed multicellular layers instead of forming large spheroids that protect them from shear stress.

Extracellular Matrix (ECM)

Approaches are aimed to reconstruct the ECM from its components in order to create a microenvironment closer to the in vivo situation, thereby resembling hepatocyte–hepatocyte and hepatocyte–ECM interactions that are critical for liver cell function.

Some bioreactors use proteins (type I collagen, fibronectin, laminin); others use proteoglycan or polysaccharide (alginate) gels to replace the natural ECM. The use of these matrix gels has been shown to enhance attachment and promote differentiation and polarization of primary hepatocytes; however, these gels reduce oxygen and carbogen diffusion and increase the hydraulic resistance in the cell compartment (9).

A probable solution of these problems was proposed by McClelland et al. (68) and Parsons and Coger (82), creating micropathways by providing the incorporation of porous and hollow polystyrene microspheres (0.55 μm in diameter) into a collagen type I gel. The microspheres form a gap between the surface of the microsphere itself and the surrounding material through which oxygen may be transported and may proceed through the pores of the microspheres. The increased diffusivity of oxygen can be explained by the increased surface area due to the microspheres added to the gel. The incorpora-

tion of cells in these transport-enhanced ECM substitute also showed protection from exposure to hypoxia and hyperoxia.

Another way of approaching the problem of adequate oxygenation of the gel was to add an oxygenated perfluorocarbon (PFC) emulsion (previously oxygenated) to a type I collagen gel. The PFC will release oxygen to adherent or embedded cells in two different ways: the carrier will initially release a bolus of oxygen stored in the gel, useful during cell attachment, and will allow the spreading of the gas when oxygen demand is highest. However, these materials have high costs, low availability, and their composition is variable from batch to batch.

As alternative, synthetic polymeric materials are attractive because of their reproducible composition and their well-defined and characterized nano- and microstructure. Moreover, they have many similarities with biomembranes regarding selective molecule transport, thermal and mechanical resistance, protection, biocompatibility, elasticity; they can easily be mass produced with modulated morphological and physicochemical properties for specific applications.

De Bartolo et al. (17) developed a membrane from a polymeric blend of modified polyetheretherketone (PEEK) and polyurethane (PU) as support for hepatocytes culture, combining advantageous properties of both polymers and membranes (permeability, selectivity, and well-defined geometry). They demonstrated that human hepatocytes, cultured for 19 days on PEEK–PU membranes, showed adhesion efficiency and functions (urea and albumin synthesis and biotransformation of diazepam) comparable with that of cells cultured on natural substrates such as collagen (89).

Chu et al. (14) developed a multilayer radial-flow bioreactor, in which nanofiber scaffolds were introduced to mimic the topography of ECMs and grafted the galactose into nanofibers to mimic the biochemical environment of ECMs. Many studies have shown that the asialoglycoprotein receptors (ASGPRs) on the surface of hepatocytes selectively adhere to galactose ligands and this interaction is able to induce the formation of hepatocyte aggregates with higher levels of liver-specific functions.

Vinci et al. (109) used biodegradable polymers composed by 20% poly-DL-lactide-*co*-glycolide (PLGA) and 10% poly-L-lactide (PLLA) solutions in chloroform, with excellent deposition characteristics in terms of viscosity and surface tension. They demonstrated that 3D scaffolds promote HepG2 proliferation and the final cell densities are significantly higher than on 2D surfaces. In the second part of the experiment, the scaffolds were placed in the multicompartment modular bioreactor (MCmB), made of polydimethyldisiloxane, having the same dimensions as a 24-microwell plate. 3D HepG2 cultures in a dynamic environment showed metabolic activities more efficient than in static monolayer hepatocyte cultures; in addition, the interstitial-like flow was identified as a critical factor in their system.

Cocultures

Within the liver parenchyma hepatocytes interact with other nonparenchymal cells (NPCs), and this communication process, taking place by means of cell–cell and cell–matrix interactions and soluble signaling mechanisms, plays a prominent role in the maintenance of differentiated functions. In addition, the preservation of liver homeostatis is also influenced by signals deriving from mechanical shear stresses to the endothelial cells by the bloodstream (73).

In particular, endothelial cells are important for the reorganization of hepatocytes in culture by secreting cytokines, nitric oxide, and matrix components; Kupffer cells mediate inflammation and innate immunity through secretion of signaling molecules and antigen presentation; stellate cells store vitamin A, maintain the extracellular matrix, modulate inflammatory response after shift to myofibroblasts (91), as well as synthesize hepatocyte growth factor (HGF). Hirose et al. (49) and Harimoto et al. (48) demonstrated that coculture with endothelial cells showed maintenance of cell junctions, hepatocyte morphology, and secretion of extracellular matrix better than hepatocytes alone. Guillouzo et al. (46) has shown that rat hepatocytes, mixed with liver epithelial cells, survived for several weeks, preserved phase I and II enzyme activities and taurocholate uptake. Novik et al. (76) has recently shown that coculture with NPCs can maintain metabolic competency for the majority of cytochrome P450 enzymes and phase II enzyme UDP-glucuronosyltransferase (UGT) as compared to static systems and monoculture flow conditions over 6 days. In addition, this group addressed the importance of assessing the hepatic clearance of drugs in a microfluidic culture system (HμREL®). They compared the intrinsic clearance rates from static culture controls with those from HμREL® device and showed that they are comparable with in vivo data from literature (12).

Geir et al. (38) has demonstrated that cocultivation of freshly isolated porcine hepatocytes with nonparenchymal cells in the bioartificial system augments specific cell functions, such as drug metabolism (cytochrome P450 activity), clearance of aggregated gamma globulin, and synthesis of albumin, and is responsible as well for ammonia release into the blood circulation, postulating the liver NPCs as a new source for ammonia production (38). In addition, primary rat hepatocytes cocultured in flat-plate bioreactor with J2-3T3 fibroblasts, as demonstrated by Allen et al. (4), is a model that represent a

novel means of exploring liver zonation and its implications with regard to enzyme induction, drug metabolism and result: CYP2B and CYP3A expression in a zonal pattern in vivo is depending on gradients of oxygen, nutrients and hormones (4). Coculture with nonhepatic cells has also been shown to be effective (41). Recently the utilization of microfabrication techniques (55) to localize cell populations in patterned configurations on rigid substrates is able to better guarantee controlled interactions in hepatocytes/fibroblast cocultures and to improve liver-specific functions and long-term viability in toxicological studies (3,4,7).

Coculture serves various purposes: induction of liver-specific functions, preservation of maximal levels of functional adhesion molecule expression, and reduction of the number of cells needed for a bioartificial liver.

Liver Cell Phenotype and Functionality

Hepatocytes are attachment-dependent cells and lose their liver-specific functions in suboptimal media, hypoxia, poor ECM composition, and after loss of cell–cell interactions.

Classic biochemical markers of liver cell function are: oxygen uptake rate (OUR), cell viability (trypan blue exclusion), metabolic synthetic activity (urea production from ammonia, albumin synthesis, glucose production from galactose), lactate consumption, detoxification (cytochrome P450 activity, phase II conjugation, and canalicular transport), and bile production. In addition, cell necrosis [leakage of lactate dehydrogenase (LDH) and aspartate aminotransferase (AST)], apoptosis (caspase3 activity), and energy status [adenosine triphosphate (ATP)] can be assessed.

The choice of new screening tests for liver function evaluation is a challenge in this field, as new tests could be able to be predictive, reproducible, sensitive, and specific. O'Brien et al. (78) developed an accelerated cytotoxicity mechanism screening (ACMS) of drugs/NCEs using freshly isolated rat hepatocytes treated with drug-metabolizing enzyme inhibitors and activators for determining in vivo rat hepatotoxicity mechanisms.

Recently, the resident time distribution (RTD) analysis has been developed to evaluate the fluid dynamics of the bioreactor (17,20) by introduction of a tracer molecule (analyzed by spectrophotometry) at the entrance of the device and recording it in time at its outlet.

Additionally, new interesting techniques have been carried out (e.g., the two-photon microscopy). Powers developed a system fabricated in two different versions, a macro- and a millireactor. A distinguishing feature of the design is that it allows replication of in situ observation of cells via light or two-photon microscopy during culture of the 3D perfused tissue structures. The calculated presence of uniform flow and nutrient conditions along the scaffold ensures a relatively consistent environment for the formation of tissue structures in each channel. The hepatic aggregates maintain their structure and viability at least 2 weeks in bioreactor culture, providing a promising platform for the studies of in vivo physiology and pathology in an in vitro environment (84).

Finally, the profile of global gene expression of the cultures and cocultures (toxicogenomics approach) is another way of assessing functionality in multiple situations: both physiologic and pathologic (e.g., after drug exposure) (56).

Long-Term Functional Stability

Cell dedifferentiation that may occur in unfavorable culture conditions is accompanied by the loss of several liver-specific functions, like cytochrome P450 monooxygenases, which affects the biotransformation capacity of the system. Accumulating loss of hepatic functions like synthetic and catabolic activities makes the cells unsuitable to compensate for the failing liver-specific functions of patients with acute liver failure.

In this overview the range of time used for evaluation of bioreactor functions started from 3–5 days (14,54,80) and went up to 14 (5,57,84), 18 (17,97), and 28 days (87,104).

Miranda et al. (70) demonstrated that the encapsulation of rat hepatocyte aggregates (100–500 μm) in high-viscosity alginate matrices and their cultivation in a dynamic bioreactor is able to maintain inducible hepatocyte phenotype with improved functional expression in vitro, promoting oxygen-dependent processes, such as urea and albumin synthesis and phase I biotransformations, for longer than 1 month.

High Throughput Systems

The essential characteristics of a high throughput system can be described as follows:

- Miniaturization and automatization of the system and the assays (volume reduction with a relative increase in surface area)
- Addition of various processes of parallelization, integration, and automation in a less expensive manner
- Possibility to conduct many tests with minimal amounts of reagents and scarce cells or tissue material, thereby facilitating rapid and parallel testing
- Capacity to closely resemble the features of liver and the fluid distribution in perfused organs
- 3D structure
- Scalability
- Standardization and reproducibility of analyses
- Organization in glass microscope slides, with the aim to allow continuous visual investigation, automated chip scanning for preparation/transport/analysis of the

kinetics and dynamics of drugs or reagents, and dynamic control of cell culture and nutrient supply. Among them, the SlideReactor, described by Schwartlander et al. (94), is a miniaturized, HF-based bioreactor system that enables continuous microscopic observation
- Choice of biocompatible materials
- Construction of simple and cheap glass slide substrate
- Last but not least, cost-effective

Bioactive Mass for Bioartificial Liver Support

Bioactive mass and cell type play a key role for clinical application of bioreactor systems. One of the unsolved questions in BAL research is with which functions and at what level a BAL should compensate for the diseased liver. It is known from partial hepatectomy studies that about 20% of a healthy liver mass, which contains approximately 200 g or 20×10^9 hepatocytes, is needed to survive. Thus, if no active liver mass is left in the patient, approximately 20 billion well-functioning hepatocytes are theoretically needed to keep the patient alive. In fact, a higher number of cells is possibly needed, because of impaired in vitro function compared to in vivo and membrane-based reactors create mass transfer restrictions. The inclusion of gels, beads, or other materials intended to stabilize cells provides additional diffusion resistances.

A variety of cell masses are used in the different BAL systems. Cell mass used in the current, clinically applied systems ranges from 5×10^9 cells (HepatAssist) to 600 g (LSS and MELS). Other systems use about 10–20 billion hepatocytes, which is, in theory, sufficient to support a liver with limited residual function. However, the viability and function of the cells prior to loading and after culture in the bioreactor may vary considerably and influence the effective biomass.

In one study the source of primary human hepatocytes for BAL use came from discarded donor livers (92). However, these cells are characterized by heterogeneity and low viability (92). Other sources of human hepatocytes are cell lines from human liver tumors, differentiated stem cells, or (conditionally) immortalized hepatocytes: to date none of these cell lines show sufficient liver specific function (10,19,86,100,108).

An important challenge to be overcome is that human liver cell lines after proliferation keep or regain their differentiation grade to be able to perform liver specific functions as close as possible to the physiological ones. These functionalities can be subdivided into protein synthesis (albumin and coagulation factors), intermediate metabolism (carbohydrate, amino acid, and fat metabolism), and detoxification (mixed function oxidase, urea synthesis, bile formation).

However, in all liver cell lines tested so far, the in vitro differentiation is far less than that of primary human hepatocytes. The ELAD system is based on C3A cells, which display a number of liver functions, such as albumin production, but, for example, their ammonia-reducing capacity is very low. More insight in differentiation-promoting factors and the influence of matrix and coculture conditions is needed.

Miniaturization of Liver Cell Culture Devices

Nowadays various strategies have been developed to obtain microfabricated bioreactors. Microfluidics can be defined as the study of flows of simple or complex fluids in microgeometries. Typical volume scales in microfluidic systems are 100 nl to 100 μl and characteristic feature sizes can range from 1 to 1000 μm. Such systems typically exhibit laminar flow, similar to the environment in vivo, and enable realization of microfluidic channels with larger surface-to-volume ratios suitable for oxygen and nutrition supply and obtainment of a controlled mixing of reagents that are limited only through diffusion.

The principal characteristics of microfluidic biochip systems can be summarized as follows (6), allowing:

- rapid, easy, and low-cost sample preparation
- proper control of microfluidic flows for dynamic cultures by continuous feeding of nutrient and waste removal
- adequate management of physiological contact times of diluted molecules with tissues and cells
- addressing multiple fluids in a controlled manner and small quantity
- measurement of kinetics and dynamics of drugs and molecules in real and very short times or over long-term period, both for acute or chronic toxicity evaluation

Microsystem technology has been used to fabricate 2D or 3D culture devices by using different types of materials, like silicon, silicone elastomer, and biocompatible and biodegradable polymers.

A silicon elastomer, like polydimethylsiloxane (PDMS), as Leclerc et al. (59) showed, is a favorable material because of its biocompatibility and high gas permeability. Primary adult rat hepatocytes, with a density of $3–4 \times 10^7$ cells/cm^3, cultured in PDMS membrane (5×5 μm hole sizes) as scaffold, showed in 15 days of perfusion: good attachment, seven and two times increases in the albumin secretion, and a sevenfold increase in ammonium removal compared to static cultures in tissue culture-treated dishes and in cultures in inserts with the same polyester membranes (79).

Another very interesting material is the polyethersulfone (PES): hollow fiber membranes described by De Bartolo et al. (17) that show high permeability and provide unhindered transport of molecules, which result in a facilitated and efficient removal of molecules from cell

compartments through a predominantly convective mechanism.

The most frequently technique used to fabricate microfluidic biochips is through replica molding of polymers. The most used polymers include polycarbonate (PC), polymethylmethacrylate (PMMA), and polydimethylsiloxane (PDMS). In addition, hybrid biochips could be fabricated using plastic for cell culture and glass or silicon, rendered biocompatible by adding extracellular matrix proteins [fibronectin, collagen, laminin, poly ethylene glycol (PEG)]. Biochips that employ microfluidic flows and fluid handling may take into account the vascular conditions found in vivo, may promote the cellular 3D liver cell organization, and allow control over shear stresses.

Other interesting systems are the so-called 'liver on chip" devices reported by Baudoin et al. (6) by using the microfluidic technology. These systems consist of microchambers containing engineered tissue and living cell cultures interconnected by a microfluidic network. Hepatoma-derived cell lines (e.g., new human hepatoma cell line, named HepaRG, derived from a human hepatocellular carcinoma) actively proliferate, displaying at confluence hepatocyte-like and biliary-like epithelial phenotypes (43); HepG2 cells (human liver carcinoma cell line), cultivated in microfluidic biochips, create "3D like structures," growing over the microchannel walls up to 2 weeks (59).

An extension of these systems, aimed to miniaturize and simplify the whole body, is the so-called Micro Cell Culture Analog (μCCA), developed by Viravaidya. μCCA or "animal-on-a-chip" is a simple four-compartment model (lung–liver–other tissue–fat). The device is fabricated from a silicone substrate using standard lithography techniques and enclosed between two Plexiglas pieces. Two chambers (lung–liver) contain living cells, whereas the "other tissue" and "fat" compartments have no cells, but mimic the distribution of fluid in rapidly and slowly perfused tissues. A fluorescent-based oxygen sensor is integrated into the system to investigate the adequacy of O_2 transfer in the system operating with cells (110).

The near future will be aimed at developing a family of microfluidic biochips to miniaturize and simplify the whole body (42). An attempt to follow this approach was performed by Li (65) with the integrated discrete multiorgan cell culture (IdMOC) system, based on the "wells within a well" concept. It consists of a cell culture plate containing wells within each of which are multiple smaller wells where cells are seeded from multiple organs: one organ per well, each in its specialized medium. This system has been successfully used to determine organ-specific toxicity using appropriate end points. The interconnection of the multiple cell types in the IdMOC model presents two advantages: it ensures that all cell types are treated under virtually identical conditions; in addition, it allows metabolites from one cell type to interact with a different cell type. Moreover the physical separation of the cell types mimics the in vivo condition, where organs are also physically separated from each other.

Another interesting system is the "Cell-on-Chip" device, developed by Lemaire et al. (64), composed of a hydrophobic perfluoro-octyl-silane (FDTS) molecular monolayer (a few nanometers thick) deposited in a patterned fashion into a hydrophilic glass layer. The spot pattern is complemented by a metallic mesh on the slides for accurate positioning of the automatic microscope with Pathfinder software. The main characteristics of this device are: the miniaturization of parallel cell-based assays using nanodrops for high throughput screening; the possibility to perform a multiplexed screening of chemicals; the sequential spotting during 5 days and automated chip scanning and smart image captures; the High Content Analysis (HCA) and data management; and, finally, the choice of a cheap and simple glass slide substrate. However, a relatively high level of variability in individual cell responses to toxic insult has been observed (45,64).

Dash et al. (16) and Domansky et al. (23) reported a liver tissue-engineered perfused bioreactor used as a model in drug toxicity. It was made of a scaffold containing a matrix of 3D liver tissue units in a multiwell format mimicking the liver capillary bed and continuously perfused by culture medium. The device houses 12 isolated bioreactors each with its integrated micropump: as culture medium flows, oxygen is consumed resulting in a gradient across the tissue similar to the oxygen gradient in the in vivo liver sinusoid. In the more recent version several reactor chambers were integrated with their pumping systems in a single plate for increased throughput, reliability, and ease of use.

Finally, Lee and Dordick (62) and Lee et al. (63) proposed the DataChip, a microarray consisting of MCF7 (human breast adenocarcinoma, estrogen receptor positive, cell line) or Hep3B (human hepatoma) cells encapsulated in a 3D hydrogel matrix, such as collagen or alginate, seeded within the matrix material and spotted onto a functionalized glass microscope slide. The DataChip yielded accurate cytotoxicity information and was able to rapidly identify metabolic activation or deactivation of xenobiotics through the action of P450 isoforms.

CONCLUSIONS

3D systems mimic and preserve the in vivo-like environment better than 2D configurations, both for bioreactors and for high throughput systems.

The equilibrium among medium flow, oxygen supply, and protection from shear stress forces is an aspect

to be considered in order to set up hepatocyte cultures showing differentiated and specialized hepatic functions for several weeks, allowing to perform both acute and chronic toxicity studies.

Cocultures with nonparenchymal cells deserve more attention, as well as engrafted scaffolds with extracellular matrix components in order to create the same interactions as in vivo.

Microfluidics and high throughput systems will allow to face the future, when miniaturized and automatized approaches will be needed to predict hepatotoxicity of drugs or NCEs combined with easy handling and low costs.

Paramount aspects to be taken into account are the capacity of the system to be tailored to individual needs by manipulating cell types or operating conditions, as well as the importance to dispose of a system that can provide visualization of cellular level events and morphological changes in a 3D tissue context.

The improvements in material biocompatibility, density, and type of cell culture and in mass transfer will lead to bioreactor designs ready for bioartificial liver applications. Such bioreactors will be able to reproduce the hepatic physiological hemodynamics and geometry in order to maintain healthy, high-functioning hepatocytes.

Although several novel and adequate bioreactor devices are available, which can be scaled up to a large (>10 billion) number of cells, the clinical applied bioartificial liver is still waiting for the optimal human liver cell line (1).

New perspectives are now coming from the fields of genomics, proteomics, and metabonomics that are able to investigate differential expression of genes, proteins, and metabolites, respectively, for toxicity and metabolism studies. However, progress towards the discovery of the ideal predictive model for drug toxicity resides in a system that globally represents the population, but individual genomic expression will also determine individual reactions to drugs.

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