has been approved by the FDA as a blood substitute for heart surgery: The fully oxygenated perfluorocarbon is infused at the time of coronary angioplasty to provide oxygen delivery during this surgical procedure. Even though the perfluorocarbon reduced cardiac trauma and pain during surgery (*5*), its use has never been fully embraced by physicians. Currently, Oxygent, a perfluorocarbon blood substitute developed by Alliance Pharmaceuticals, is in stage II/III clinical trials in the United States.

Risk Versus Benefit

New testing and screening procedures have rendered the donor blood supply increasingly safe. For example, the risk of transfusion-associated HIV infection is now estimated to be as low as 1 per 835,000 transfused patients (*8*). Similarly, the risk of transfusion-associated infection with hepatitis C virus (HCV) is between 1 per 300,000 and 1 per 600,000, compared with an incidence of 1 per 103,000 in the early 1990s before a test for HCV became available. As the safety of the donor blood supply continues to improve, there must be careful consideration of the advantages of blood substitutes over donated blood, particularly given that new blood substitutes potentially carry unknown risks. Yet a shortage of donor blood for transfusion still advocates for the development of readily available blood substitutes. However, there is still a long way to go before artificial blood can replace real blood in routine transfusions. Most important, the intravascular dwell times of blood substitutes need to be increased, the cost of these products needs to be competitive, and difficulties with obtaining and processing sufficient amounts of these compounds must be overcome.

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VIEWPOINT

A Bioartificial Liver—State of the Art

Alastair J. Strain* and James M. Neuberger

End-stage liver disease is treated by liver transplantation, but donor organ shortages remain a serious problem. This has prompted the design of bioartificial liver devices to "bridge" patients until they either recover or receive a liver transplant. In these devices, patient plasma is circulated extracorporeally through a bioreactor that houses liver cells (hepatocytes) sandwiched between artificial plates or capillaries.

The healthy liver is able to regenerate itself after acute injury, but once damaged by fibrosis and cirrhosis—caused by a variety of chronic conditions such as alcohol abuse or infection with hepatitis virus B or C—it can no longer regenerate normally (*1*). Liver transplantation is a routine treatment for end-stage liver disease, but donor organ shortages remain a serious problem. Many patients with acute liver failure die while waiting for a transplant, and those with chronic disease often deteriorate so much that their survival rate after transplantation is low. Remarkably, "supporting" acute liver failure patients until their own liver repairs itself may negate the need for a liver transplant (*2, 3*). This has generated interest in designing a "bridging" device that would support or replace normal liver function until the patient's own liver recovered or a donor liver became available for transplant. Alternatively, a bridging device could support a failing liver long-term, in the same way that dialysis supports the failing kidney (*2, 3*). The principal goal is to develop a bioartificial liver (BAL) device in which patient plasma is circulated extracorporeally through a bioreactor that houses metabolically active liver cells (hepatocytes) sandwiched between artificial plates or capillaries.

What must a BAL bioreactor accomplish? The liver has a number of crucial functions that are principally carried out by hepatocytes. These cells synthesize many proteins, including clotting factors; they produce bile and regulate carbohydrate, fat, and protein metabolism; they detoxify the ammonia product of nitrogen metabolism and break down alcohol and drugs. In addition, the liver has Kupffer cells that are part of the immune system. The problem is deciding which liver functions are the most important and should be carried out by the BAL bioreactor. Biosynthetic capacity is perhaps the least essential because most proteins synthesized by the liver can be given exogenously to patients. The generation and detoxification of ammonia is undoubtedly important. Although an increase in ammonia in the blood is toxic to the central nervous system, reduction of ammonia levels per se is not sufficient to alleviate this toxicity (*4*). In response to this panoply of different functions, researchers are attempting to develop BAL devices in which hepatocytes are optimally maintained so that they carry out as many activities as possible.

The Best Type of Cell for a BAL Bioreactor

Constituting 70% of the cellular content of the liver, the primary hepatocyte is clearly the cell of choice for a BAL bioreactor, particularly as it carries out most of the liver's functions. However, when removed from the complex architecture of the liver, hepatocytes rapidly lose liver-specific gene expression and become phenotypically unstable in culture. By manipulating tissue culture conditions—providing an extracellular matrix and growth factors, and coculturing hepatocytes with other types of liver cells—much can be done to maintain hepatocyte stability in vitro (*5*). Indeed, cross-talk among hepatocytes (which form the liver parenchyma), nonparenchymal liver cells, and bile duct epithelial cells is important for optimal hepatic activity (*5*) (Fig. 1).

The enormous scale-up required to use BAL devices clinically is problematic: At least 10^{10} hepatocytes in a BAL bioreactor would be needed to support a patient's failing liver. Given that the proliferative capacity of primary human hepatocytes and the ability to cryopreserve them is limited, there is a serious numbers problem (*5*). In short, there is insufficient good-quality human liver tissue from which to derive hepatocytes for routine clinical use in a BAL device. One alternative is to use hepatocytes from other species, par-

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ticularly pigs. However, there is still an ongoing debate about the risk of zoonoses, animal infections transmitted by cells from other species (xenogeneic cells) (*6*). Porcine-derived hepatocytes are being used in clinical studies of BAL devices in the United States, but such studies remain on hold in the U.K. and most other European countries.

Using cultured hepatic cell lines overcomes the human hepatocyte shortage, although it is still hotly contended whether the hepatic lines currently available retain sufficient functional capacity for use in BAL devices. One solution may be to combine several different liver cell lines in the bioreactor and thus exploit their functional complementarity. Alternatively, it should be possible to genetically engineer hepatic cell lines to have the desired capabilities. For example, one recent study reported reversible immortalization of a human hepatocyte line using the Cre-Lox genetic engineering system, which allowed excision of the immortalizing gene, thus removing the threat of tumor formation (*7*). The safety aspects of using genetically altered and potentially tumorigenic liver cells in the clinic still needs to be addressed. Despite these reservations, one experimental program is clinically testing a BAL device containing the C3A hepatocyte line, a subclone of the ubiquitous HepG2 hepatoblastoma cell line (*8*).

Liver stem cells, or even stem cells from other tissues, could potentially provide an alternative source of human hepatocytes. In addition to bone marrow, stem cells reside in adult tissues such as the liver and central nervous system, and have much greater plasticity than previously realized (*9, 10*). Investigators are trying to define the factors controlling the differentiation of stem cells into the cell type of choice. The key will be to determine conditions under which stem cells can be clonally expanded in an undifferentiated state and then quickly "switched" to the desired phenotype. Although the differentiation of bone marrow–derived stem cells into hepatocytes has been demonstrated in vivo (*11–13*), it has not been achieved in vitro as yet. However, liver-derived hematopoietic (blood) cells can give rise to cells that resemble bile duct epithelial cells (*14*). Embryonic stem (ES) cells offer another valuable option, particularly as human ES cell lines are now available (*15–17*). Differentiation of human ES cells into cardiomyocytes and neural progenitor cells has been reported, taking us a step closer to the reality of using them as a viable source of human hepatocytes. Just as vital as finding a suitable source of hepatocytes is the need to develop a workable culture system where hepatocytes (wherever they come from) can be optimally maintained on a large scale.

Designing a BAL Bioreactor

The basic BAL bioreactor consists of a column containing a collection of hollow-fiber capillaries through which patient plasma is pumped (Fig. 2). Hepatocytes are inoculated into the extracapillary space (*8, 18*) either alone or attached to microcarrier beads (*19*). In the secondary circuit, patient (or animal) plasma is separated, warmed, oxygenated, and then perfused through the lumen of the bioreactor capillaries (Fig. 2). This allows free exchange of molecules between hepatocytes and patient plasma: Hepatocytes extract oxygen and nutrients and detoxify chemicals in the plasma, and their metabolites pass into the plasma. The ideal BAL design must ensure optimal ex vivo maintenance of hepatocytes. It has been assumed, but not yet proven, that simple "flow-through" BAL systems achieve this. However, given that conventional monolayer cultures cannot optimally maintain hepatocytes, it is likely that hepatocytes will need to be induced to form cellular aggregates in which they reacquire their polarization (that is, the asymmetric localization of surface proteins that enables different parts of the cell to carry out different functions).

A more sophisticated BAL bioreactor has been developed in Berlin by Gerlach and colleagues (*20*). This bioreactor comprises three sets (bundles) of interwoven capillaries that create a three-dimensional extracapillary space in which to house hepatocytes. One set of capillaries provides an efficient oxygenation supply, and the remaining two sets control inflow and outflow of plasma, respectively. This BAL bioreactor provides an optimized compartment for hepatocytes that encourages them to reorganize into functional cellular aggregates (*20*). The downside is that the Gerlach bioreactor is extremely complex to run. Another design by Chamuleau and colleagues (Amsterdam) incorporates a spirally wound polyester matrix sheet that includes an integral hollow-fiber compartment for oxygenation (*21*). This design again creates an environment that in theory is more amenable to the optimal maintenance of hepatocytes, enabling them to retain many of their functions. Together with the Sussman (*18*) and Demetriou (*19*) systems, the Chamuleau and Gerlach BAL devices are being tested in clinical trials. Other BAL bioreactor designs under development include a flat-bed capillary arrangement that incorporates a collagen gel system to support hepatocytes, and a series of flat stacking plates or other radial flow elements (*22–24*). Still other BAL bioreactors are under development in the laboratory (*2, 3*) but are not yet

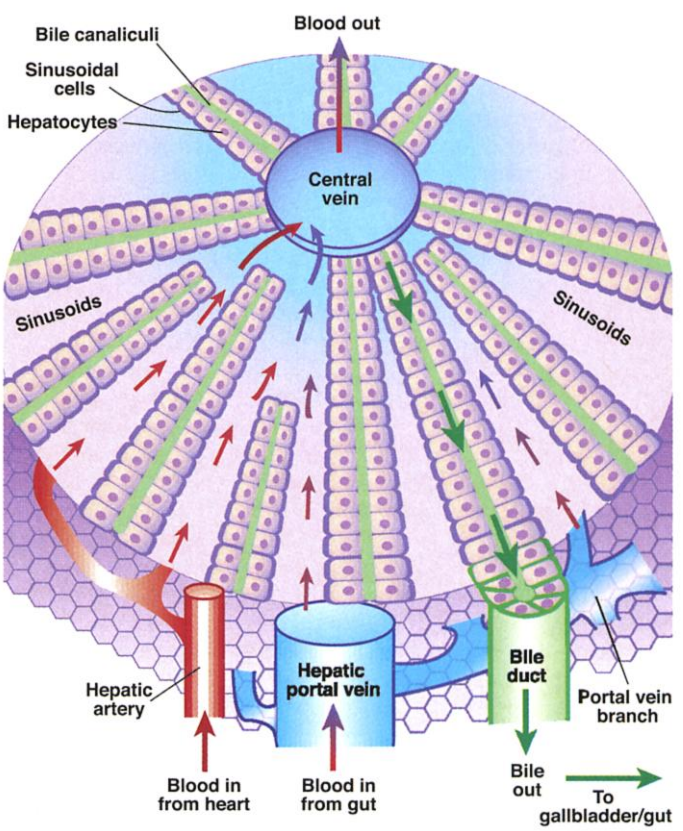

Fig. 1. Cellular architecture of the liver. Liver epithelial cells called hepatocytes (pink) are arranged in cords between the capillaries (sinusoids) of the liver. Oxygenated blood enters the liver from the heart via the hepatic artery (red) and from the gut via the hepatic portal vein (blue), mixes in the sinusoids, and drains via the hepatic central vein back to the heart. Sinusoidal cells (purple)—including endothelial cells, Kupffer cells, and stellate cells—line the sinusoids, thus separating hepatocytes from blood. The hepatocytes are only exposed to plasma, allowing exchange of plasma proteins, nutrients, and metabolites. Bile (dark green arrows) is synthesized by hepatocytes and secreted into small channels (canaliculi; pale green) between adjacent cells. The bile collects in ducts formed by biliary epithelial cells and drains from the liver into the gallbladder (or, if the gallbladder has been removed, bile drains directly into the gut). The challenge of a BAL bioreactor is to attempt to partly recreate the complex tissue architecture of the liver with all of its myriad functions. [Illustration: Nathalie Cary]

sufficiently advanced for testing in the clinic.

There is an enormous literature (too extensive to review here) describing the testing of BAL devices in vitro and in animal models (*2, 3, 8, 18, 19*). Many different parameters have been measured as indicators of hepatocyte viability and function, including secondary endpoints such as albumin or clotting factor production, urea synthesis, conjugation of the bile pigment bilirubin, drug metabolism, and a variety of other metabolic processes. BAL bioreactors have been tested in a number of different small and large animal models of liver failure. For example, Flendrig *et al.* recently reported prolonged survival of pigs whose livers were subjected to severe ischemia by surgical manipulation of the blood supply (*25*). In a carefully controlled study, ischemic animals were treated with the Chamuleau bioreactor system housing 1.4×10^{10} porcine hepatocytes. Blood ammonia and bilirubin levels decreased significantly and the longest surviving animal was kept alive for 63 hours (*25*). Thus, there is no doubt that hepatocytes can and do function satisfactorily in various BAL systems, but interpreting the data from experimental studies and extrapolating them to the clinic has proved difficult.

Clinical Trials

The first clinical use of a BAL bioreactor in 1987 was to treat a single patient with acute liver failure. The patient's serum bilirubin levels fell, his neurological condition improved, and he was discharged from hospital, thus demonstrating the potential value of the approach (*26*). This BAL device consisted of an adapted artificial kidney dialysis unit loaded with cryopreserved rabbit hepatocytes (*26*). Despite the huge difficulties associated with designing and running clinical trials and the problems of selecting clinically relevant endpoints, some successful treatment regimens have been reported. The most promising clinical results are those of Sussman's group with the C3A human hepatoma cell line, and of Demetriou and colleagues with primary porcine hepatocytes, each using their own BAL bioreactor (*27, 28*). Ideal patients for treatment are those suffering from acute liver failure, who need to be "bridged" until they can receive a liver transplant or until they recover (*2, 3, 27, 28*).

Although the safety and feasibility of BAL devices were quickly established, demonstrating efficacy has been much more difficult. We await the outcome of randomized, controlled clinical trials. The first such trial, consisting of 24 acute liver failure patients (12 treated with the Sussman BAL device containing C3A cells, and 12 controls), has been completed in the U.K. (*29*). The safety and feasibility of the BAL bioreactor was demonstrated, but there was no clinical survival benefit. This result could be at least partly explained by a higher than predicted survival in the control group. Clearly, increased patient numbers offer the only means of resolving the question of efficacy. A larger multicenter trial, again with the C3A cell line, is underway in the U.S. and U.K. (under the direction of Vitagen).

Results of a second, more extensive clinical trial with Demetriou's porcine hepatocyte system were recently reported at the American Association for the Study of Liver Diseases meeting in Dallas (*30*). This major multicenter trial (supported by Circe Biomedical) includes more than 170 patients, most of whom have acute liver failure. This study is important because it is the first large-scale trial conducted in both U.S. and European centers that attempts to demonstrate efficacy. Although preliminary results are described as encouraging, a detailed analysis of the full study awaits critical appraisal and peer review. Two other BAL systems (the Amsterdam and Berlin devices) have been tested clinically in preliminary, and as yet unpublished, clinical trials with small numbers of patients. Both BAL devices contained pig hepatocytes and were used to treat patients with acute liver failure.

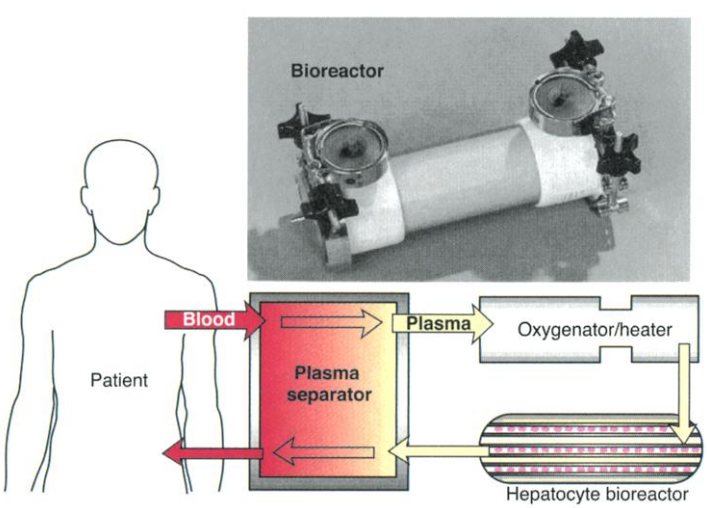

Fig. 2. A BAL bioreactor. In the primary circuit, the patient is connected by venous catheters to a plasma separator. In the secondary circuit, the plasma is then passed through a heat exchanger and oxygenator (some bioreactors include integral oxygenation fibers) before going through the lumen of the capillaries in the bioreactor column (bottom right and top image). Up to 10^{10} human or porcine hepatocytes are housed in the extracapillary spaces of the bioreactor column. Blood cells are added back to the plasma before it is returned to the patient. [Photo courtesy of R. Chamuleau]

The Future

The greatest challenge for the BAL bioreactor is how best to maintain viable functional hepatocytes outside of the body. Perhaps we can look to the biomaterials scientists (see page 1014) (*31*) to provide us with biomaterials that could induce hepatocytes to retain their epithelial polarization. In addition, tissue engineering strategies (see page 1009) (*32*) could be used to coax hepatocytes to reorganize into functional three-dimensional aggregates in vitro. Interactions among the different types of hepatic cell populations are essential for the liver to operate appropriately. Coculture of hepatocytes with nonparenchymal liver cells has been shown to be beneficial (*5*). The downside is that inclusion of other cell types would inevitably make BAL design, construction, and handling even more complex.

Bile production by hepatocytes is not normally reproduced in a BAL bioreactor. It is possible that some of the net products of bile production and metabolite excretion could be toxic to hepatocytes if not removed. Investigators tend to assume, indeed hope, that these potentially toxic intermediates, if made, are simply washed away or are sufficiently diluted that they do not affect the hepatocytes. It is certainly hopelessly ambitious to engineer bile drainage into the current generation of bioreactors. Intriguingly, it has recently been reported that bile duct–like structures form in cultures of bile epithelial cells (*33, 34*).

Even if these design challenges are overcome, we still face the cell supply problem. Looking into the future, ideally, a stem cell inoculum would be added to the BAL bioreactor of choice and allowed to expand before the appropriate molecular switches are activated, with the induction of the required hepatic phenotype and the assembly of hepatocytes and other types of liver cells into organized liver tissue.

Finally, another issue that must be considered is ease of handling. In the acute liver failure situation, a bioartificial liver machine must be available immediately without the need for lengthy preparation times. It must be

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"user friendly" for the critical care nursing and medical staffs, who are under inordinate stress when dealing with acutely ill patients and when decision times are short. These are the challenges faced collectively by bioengineers, cell biologists, and clinicians alike as we look to the future of the BAL bioreactor in clinical medicine.

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35. Supported by the Wellcome Trust and the UK Biotechnology and Biological Sciences Research Council.

VIEWPOINT

Tissue Engineering—Current Challenges and Expanding Opportunities

Linda G. Griffith[1]* and Gail Naughton[2]

Tissue engineering can be used to restore, maintain, or enhance tissues and organs. The potential impact of this field, however, is far broader—in the future, engineered tissues could reduce the need for organ replacement, and could greatly accelerate the development of new drugs that may cure patients, eliminating the need for organ transplants altogether.

Introduction

The field of tissue engineering exploits living cells in a variety of ways to restore, maintain, or enhance tissues and organs (*1*, *2*). Tissue engineering conjures up visions of organs built from scratch in the laboratory, ready to be transplanted into desperately ill patients. The potential impact of this field, however, is far broader—in the future, engineered tissues could reduce the need for organ replacement, and could greatly accelerate the development of new drugs that may cure patients, eliminating the need for organ transplants altogether.

To engineer living tissues in vitro, cultured cells are coaxed to grow on bioactive degradable scaffolds that provide the physical and chemical cues to guide their differentiation and assembly into three-dimensional (3D) tissues (*3*). The assembly of cells into tissues is a highly orchestrated set of events that requires time scales ranging from seconds to weeks and dimensions ranging from 0.0001 to 10 cm. Coaxing cells to form tissues in a reliable manner is the quintessential engineering design problem that must be accomplished under the classical engineering constraints of reliability, cost, government regulation, and societal acceptance.

Even though fewer than five engineered tissues have been approved by the Food and Drug Administration (FDA), more than 70 companies are spending a total of $600 million per year to develop new products (*2*). There are still many technical challenges to overcome before we create "off-the-shelf" tissues that represent the translation of scientific discoveries into treatments for millions of patients. The successful large-scale production of engineered tissues requires an adequate source of healthy expandable cells, the optimization of scaffolds, and the creation of bioreactors, which mimic the environment of the body and that are amenable to scale-up. Additional challenges include the preservation of the product so that it has a long shelf-life and the successful use of various approaches to prevent tissue rejection.

Biological Challenges: Cells and Their Sources

There are three principal therapeutic strategies for treating diseased or injured tissues in patients: (i) implantation of freshly isolated or cultured cells; (ii) implantation of tissues assembled in vitro from cells and scaffolds; and (iii) in situ tissue regeneration. For cellular implantation, individual cells or small cellular aggregates from the patient or a donor are either injected into the damaged tissue directly or are combined with a degradable scaffold in vitro and then implanted. For tissue implantation, a complete 3D tissue is grown in vitro using patient or donor cells and a scaffold, and then is implanted once it has reached "maturity." For in situ regeneration, a scaffold implanted directly into the injured tissue stimulates the body's own cells to promote local tissue repair.

Sources of cells for implantation include autologous cells from the patient, allogeneic cells from a human donor who is not immunologically identical to the patient, and xenogeneic cells from a different species. Each category may be further delineated in terms of whether the cells are adult or embryonic stem cells (capable of both self renewal and differentiation into a variety of cell lineages), or a mixture of differentiated cells at different stages of maturation (including rare stem and progenitor cells). Some approaches use cell mixtures, whereas others rely on separation or enrichment of stem cells.

Although the prospect of using xenogeneic cells for tissue repair remains controversial because of the potential for transmitting animal pathogens to humans, xenogeneic cells could perhaps temporarily support an ailing tissue until either a human donor organ becomes available for transplant, or the tissue repairs itself. For example, pig liver cells (hepatocytes) grown in extracorporeal biore-

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