REVIEW

Cell choice for bioartificial livers

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Abstract. It is unlikely that human hepatocytes can be isolated on a scale sufficient to treat more than a fraction of the patients who need bioartificial liver (BAL) treatment. The use of animal cells results in the concerns related to the transmission of infectious pathogens and immunologic and physiologic incompatibilities between the donor and humans. Human embryonic stem cells and bone marrow multipotent adult progenitor cells have received great attention as a possible source for BALs. The use of tightly regulated clonal hepatocyte cell lines would be attractive. Such cell lines grow economically in tissue culture and provide the advantage of uniformity, sterility, and freedom of pathogens. In this paper, the authors review the choice of cells for BALs and discuss our reversible immortalization system of human liver cells using a retroviral transfer of immortalizing genes and subsequent Cre/loxP-mediated site-specific recombination. (Keio J Med 52 (3): 151–157, September 2003)

Key words: bioartificial liver, porcine hepatocytes, stem cells, reversibly immortalized human hepatocytes

Introduction

Acute liver failure (ALF) is often life threatening and dramatically diminishes the quality of life of patients.[1] Orthotopic liver transplantation has become a successful therapy for ALF, but this procedure is highly costly, limited by the scarcity of donor livers and associated with high morbidity and mortality.[2] There is a compelling need for developing effective alternatives for patients with ALF. Considering the potential of the liver to regenerate, temporary support with bioartificial livers (BALs) is an attractive approach.[3,4] Since technologies of tissue cell culture and biomaterials have been greatly advanced, many designs of BALs, including (1) a biological component, (2) a bioreactor, and (3) a whole blood or plasma perfusion system are currently under investigation. In the present review, we focus on cell choice for developing BALs.

In vitro and in vivo experiments for BAL development

As shown in Tables 1 and 2, many researchers have made great efforts to develop BALs using various types of cells in different modules.[5–43] For *in vitro* experiments rat hepatocytes were favorably used and pig hepatocytes were often utilized for *in vivo* BAL studies. Considering BALs developed for humans, possible cell choice is limited, including normal human adult and fetal hepatocytes, *in vivo* transformed human hepatocytes, porcine hepatocytes, human-derived stem cells, and reversibly immortalized human hepatocytes.

Normal human hepatocytes

Although normal human livers are an ideal source of cells for BAL therapy, donor liver shortage is severe world-wide and the availability of the liver for hepatocyte isolation is unfortunately limited by competition for the use in whole organ transplantation. Strom *et al.* demonstrated the usefulness of human fetal hepatocytes for cell therapies because of their proliferative capacity and differentiation potential, but the use of the fetal hepatocytes raises an ethical issue.[44] On the basis of results from liver surgery in humans, it is estimated that approximately 10% to 30% of residual liver parenchyma would be required to support the life of

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Table 1 *In Vitro* BAL Experiments

Investigator	Cell type	Evaluation
Wolf and Munkelt[5)]	Reuber hepatoma cells	Bilirubin conjugation
Hager[6)]	Mouse	Ureagenesis, protein synthesis, diazepam metabolism
Kasai[7)]	Dog	Maintenance of ATP
Demetriou[8)]	Rat	Bilirubin synthesis and conjugation, protein synthesis, diazepam metabolism
Jauregui[9)]	Rat	
Yanagi[10)]	Rat and rabbit	Ammonia removal, urea synthesis, cyclosporine metabolism
Moscioni[11)]	Human	
Shatford[12)]	Rat	Albumin synthesis, amino acid and lidocaine clearance
Sussman and Kelly[13)]	C3A cells	Glucose utilization, albumin synthesis
Nyberg[14)]	Rat	Synthesis of albumin and urea, lidocaine metabolism, arginine clearance
Li[15)]	Rat	Urea, albumin synthesis
Rozga[16)]	Rat	Cyclosporine and 19-nor-testosterone metabolism, bilirubin conjugation
Fremond[17)]	Rat	
Kong[18)]	Pig	Lidocaine, ethoxyreso-rufin metabolism
Bader[19)]	Rat	Cyclosporine and rapamycin metabolism
Gerlach[20)]	Pig	Amino acid and keto-acid metabolism
Morsiani[21)]	Pig	Cholate metabolism
Kobayashi[22)]	Immortalized human hepatocytes	Clearance of NH3
Linti C[23)]	Pig	P450-dependent metabolic function (MEGX test)

Table 2 *In Vivo* BAL Experiments

Investigator	Cell type	Evaluation
Matsumura[24)]	Rat	Albumin synthesis
Olumide[25)]	Pig	Neurologic improvement
Kasai[7)]	Dog	Improved survival
Uchino[26)]	Pig	Improved survival
Yanagi[10)]	Rabbit	Clearance of NH3
Arnaout[27)]	Rat	Bilirubin conjugation
Shnyra[28)]	Rat	Improved survival
Nyberg[14)]	Rat	Albumin synthesis
Sussman[29)]	C3A cells	Improved survival
Takahashi[30)]	Pig	Improved survival
Fremond[17)]	Rat	Bilirubin conjugation
Rozga[31,32)]	Dog, Pig	Clearance of NH3 and lactate
Gerlach[33)]	Pig	Clearance of NH3
Jauregui[34)]	Rabbit	Clearance of diazepam and lidocaine clearance, improved survival
Dixit[35)]	Pig	Improved survival
Chen[36)]	Pig	Improved survival
Suh[37)]	Rat	Improved coagulopathy and survival
Stevens[38)]	Pig	Improved survival
Mazariegous[39)]	Pig	Hemodynamics (transient hypotension and thrombocytopenia)
Millis[40)]	C3A cells	
Abrahames[41)]	Pig	Elimination of phenylephrine, reduction of dopamine and respiratory support
Ambrosino[42,43)]	Pig, Matrix	LDH leakage, NH3 clearance, urea synthesis, 7-ethoxycoumarin O-deethylase (ECOD) activity and pseudocholine esterase

patients.[45] Thus, it seems to be difficult to provide the fetal hepatocytes in such a large scale.

In vivo transformed human hepatocytes

Human hepatocytes should be ideally the optimal biological component in BALs, however, this approach is impractical due to the shortage of human livers. Sussman *et al.* used a human differentiated hepatoblastoma cell line C3A as a source of the ELAD.[3] In the literature, the C3A retains differentiated hepatic functions while showing a short population doubling time and contact inhibition. Patients underwent ELAD with whole-blood perfusion continuously for relatively long periods of time.[3] In the initial group of 10 patients, no significant effect on the outcome in patients with ALF was noted, with only one survivor. The ELAD therapy was further tested at King's College Hospital in London, with no significant evidence of metabolic support and no beneficial effect on patient survival.[13] However, leakage of these tumor-derived cells into the patient's circulation on unexpected device failure remains a major concern in such a compromised host. In fact, the extracapillary passage of HepG2 cells, which are similar to C3A, from a hollow fiber membrane was observed *in vitro* BAL experiments.[39]

Porcine hepatocytes

In 1994, Nyberg *et al.* reported that primary hepatocytes outperformed all available liver cell lines in

terms of bio-transforming functions.[46] The result prompted investigators to use porcine hepatocytes to develop BALs. In addition, pigs have similar physiology to humans and one porcine liver can provide enough hepatocytes for several BAL treatments. Demetriou *et al.* started Federal Drug Administration (FDA) approved clinical trials using a porcine hepatocyte-based BAL system (HepatAssist, Circe Biomedical Inc., Lexington, MA, USA) since 1994.[47] They have used plasma separation and BAL perfusion for 6 hours and patients tolerated the treatments well.

They have mentioned that porcine hepatocytes have a tendency to form cell aggregates, resulting in the maintenance of differentiated hepatic functions.[47] Regarding the clinical studies using xenogenic cells, the potential hazard of pathogens from porcine-derived organs raises a new issue of xenozoonosis. Concern has particulary increased regarding xenozoonotic infection, since porcine endogenous retroviruses (PERV) were reported to infect human cells.[48–50] The phase II/III trial using HepatAssist in 171 patients was conducted and the 30-day survival was reported to be 70% for a BAL-treated group and 60% for a control group receiving standard medical therapy (personal communication with Dr. Demetriou AA, Cedars-Sinai Medical Center, LA, USA). Surviving patients were retrospectively examined for the presence of PERV and there was no evidence of viral transmission from pigs to humans. The data were encouraging in developing clinical therapies using porcine hepatocytes, however, needless to say, we should collect pertinent results regarding the safety of xenogenic porcine tissues and cells in the future.

Human-derived stem cells

Regenerative medicine using stem cell biology is attracting great attention. Strategies for regenerative therapy using a stem cell system are roughly partitioned into: 1) the use of organ (or tissue) stem cells, 2) the use of embryonic stem (ES) cells, 3) the use of dedifferentiation/transdifferentiation of differentiated cells. Therapeutic designs should be determined based on a comprehensive consideration of the available information. Differentiation of human ES cells and multipotent adult progenitor cells (MAPCs) in the human bone marrow into hepatocytic cells are reviewed here. ES cells are undifferentiated stem cell lines established from the epiblasts that are present in the inner cell mass of an early embryo at the blastocyst stage. Epiblasts differentiate into the developmental process and they are capable of differentiating into the three germ layers. Thus, ES cells can be induced to differentiate into various types of cells under specific culture conditions. Human ES cell lines were established by Thomson *et al.* in 1998 and, therefore, have been a focus of regenerative medicine and received attention as a potential source for BAL.[51] Investigators at Geron Corporation (Menlo Park, CA, USA) have currently shown that human ES cells can be differentiated into hepatocytic cells *in vitro* under the presence of sodium butyrate in the culture medium.[52] The expression of albumin, cytokeratin 8, anti-alpha trypsin, and glycogen is positive in such differentiated cells. Catherine *et al.* have identified the presence of MAPCs in the mouse, rat, and human bone marrows and demonstrated that MAPCs differentiated into the functional hepatocytic cells with producing albumin and urea in the hepatocyte growth factor (HGF)- and basic fibroblast growth factor (bFGF) based culture medium.[53] These cells would be a candidate for developing BALs, however, one should keep in mind that neither transdifferentiation or dedifferentiation of such cells in BAL modules can be guaranteed. Stem cell biology should be well characterized in the near future to facilitate BAL therapies.

Reversibly immortalized human hepatocytes

Concerns about porcine hepatocytes include xenozoonosis and immunologic and physiologic incompatibility with the human host, while human cell-lines expose patients to the potential risk of releasing tumor cells or tumorigenic products from the BAL device into their circulation.[54,55] The utilization of stem cells in humans will need some time to clearly address the stem cell biological system. To overcome these problems, we have focused on reversible immortalization of human hepatocytes to intentionally control the population expansion. A tightly regulated system for cell growth should be considered to generate immortalized hepatocyte cell lines that are suitable for clinical use. Thus, we applied a Cre/loxP site-specific recombination system. DNA sequences intervened by loxP recombination targets can be excised after expression of Cre recombinase.[56] The Cre/loxP system has been widely used to control gene expression in transgenic mice. In order to provide stringent control over expression of transforming genes, we immortalized human hepatocytes with retroviral vectors SSR#69 (for expressing SV40T)[57] and SSR#197 (for expressing human telomerase reverse transcriptase (hTERT))[58] with selectable positive and negative markers, which were intervened by a pair of loxP recombination targets and subsequently excised by Cre/loxP site-specific recombination, as illustrated in Fig. 1. Using SSR#69, we established a reversibly immortalized human hepatocyte cell line NKNT-3.[59]

The ability to measure *in vitro* differentiated functions is extremely dependent on culture conditions and, for oncogene-transformed cells, there is evidence that

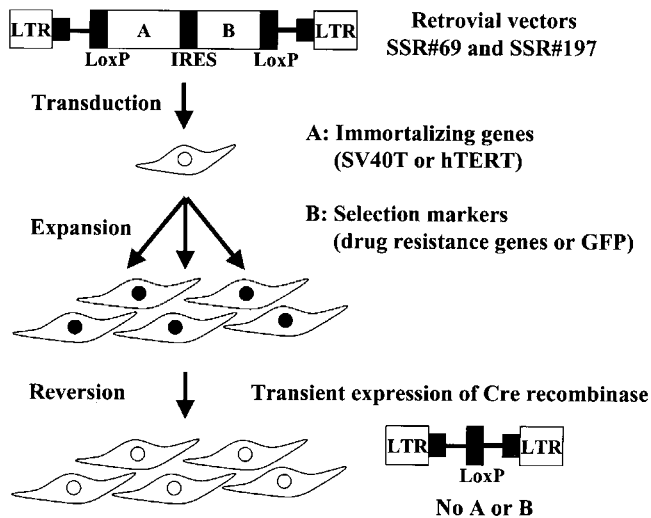

Fig. 1 Illustration demonstrating reversible cell immortalization. We utilized retroviral vectors SSR#69 and SSR#197 to immortalize human liver cells. After Cre/loxP site-specific recombination, the intervening DNA segment between the two loxP recombination targets can be excised (LTR, long terminal repeat; SV40T, simian virus 40 large T antigen; hTERT; human telomerase reverse transcriptase; IRES, internal ribosomal entry site).

an improvement in differentiated cellular responses can be accomplished simply by serially transferring the cells into animals.[60,61] Thus, we performed intrasplenic transplantation of NKNT-3 cells treated with Cre recombinase, reverted NKNT-3 cells, in a rat model of liver failure. Transplantation of the cells significantly prolonged the survival of the rats. Extension of this procedure to other cell types presenting in the human liver allows studies of cell-cell interaction and further contributes to the development of cell therapies and BALs.[62] One of our long-term goals is the development of BAL systems that closely mimic the function of the normal liver *in vivo*. Pure cultures of hepatocytes recapitulate several key liver functions but fail to provide adequate levels of a few important detoxyfying enzymes that include the cytochrome p450 associated enzymes (CYPs). Among the known crosstalk between hepatocytes and other liver cells, hepatic stellate cell (HSCs) are believed to play an essential role.[63,64] Previous attempts to develop BAL have focused on the hepatocyte biosynthetic function, ignoring the reticuloendothelial role performed by liver sinusoidal lining cells. Recently, heterotypic cell interactions between parenchymal cells and nonparenchymal neighbors have been recognized to be central to the function of many organ systems. In both the developing and mature adult liver, cell-to-cell interactions are imperative for coordinating the sophisticated liver functions.[65] Therefore, we have applied the Cre/loxP system to human liver endothelial cells and hepatic stellate cells.[58,62] We have found that the co-culture of NKNT-3 cells with SSR#197-immortalized hepatic stellate TWNT-1 cells increased CYP3A4 and CYP2C9 expression. The finding supports the contention that heterotypic cell interaction is of importance to enhance the production of liver specific enzymes by hepatocytes *in vitro*.[66,67] Because newly developed drugs are still screened for their safety and efficacy in animal models, BALs of multiple cell composition should become an attractive platform as an alternative to animal testing. Provision of the bile drainage system is also an important issue to develop novel BALs. To address the issue, we have currently established a human cholangiocyte cell line MMNK-1. The MMNK-1 cells showed the expression of cytokeratins 9 and 17 and cholangiogenic potential in a Matrigel. Development of a coculture system of hepatocytes and MMNK-1 cells is now under investigation.[68] The application of BALs that we are currently developing includes: 1) Bridge use until organ transplantation is available or hepatic regeneration is completed, 2) Prevention of hepatic encephalopathy of patients with decompensated liver cirrhosis, 3) Models for drug testing, and 4) Development of new cellular products. The use of Cre/loxP-based reversible immortalization represents an important step in the development of a potentially novel strategy for resolving the organ shortage that currently limits the use of healthy human hepatocytes for BALs. This technology may be applicable to a variety of somatic cells and could potentially be utilized to treat a large fraction of clinically significant pathologic conditions.

Redundant safeguards in the reversibly immortalized human hepatocytes

A method to protect patients from the possible migration of cells utilized in BALs is to introduce suicide genes into the cells. The cells modified to contain a herpes simplex virus-thymidine kinase gene (HSV-TK) become sensitive to the treatment with an antiviral agent ganciclovir (GCV), whereas normal cells are unaffected by the drug.[69] GCV is converted into nucleotide-like precursors that kill cells containing HSV-TK by blocking DNA synthesis. Since GCV does not interact with human thymidine kinase, it is not toxic to most human tissues lacking HSV-TK. A cytosine deaminase gene can be utilized as a suicide material.[70] 5-fluorocytosine (5-FC) at the therapeutic doses is not toxic to normal cells, but the cells expressing cytosine deaminase convert 5-FC to 5-fluorouracil (5-FU). Then, 5-FU is further metabolized to produce suppression of cell growth and cell death by inhibition of RNA and DNA syntheses.

Prospect of the future of BAL therapy

Kjaergard *et al.* have currently performed a systematic review to evaluate the effect of artificial and bioartificial support systems for acute and acute-on-chronic liver failure.[71] They have concluded that artificial support systems reduce mortality by 33% in acute-on-chronic liver failure compared with standard medical therapy and that artificial and bioartificial support systems did not appear to affect mortality in ALF. Precipitating factors in ALF include drug toxicity and viral hepatitis, which are difficult to treat. This may explain why support systems are effective in acute-on-chronic liver failure but not in ALF. Based on their review, currently available BALs cannot be effective enough in patients with ALF. Thus, it would be important to treat such patients not only with a new type of a highly functional BAL in which hepatocytes will be utilized in conjunction with non-parenchymal liver cells and extra cellular matrices but also with currently available viral therapies.

Conclusion

The goal of BAL therapy is to replace whole-liver transplantation in patients with an acutely devastated liver. Toward this goal, integration of cell culture and gene transfer technology and the cutting wedged-biotechnology is urgently required for the development of sophisticated BAL systems that mimic the *in vivo* liver.

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