Recent advances and problems of artificial liver support systems

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Introduction

The liver is the largest organ in the human body with numerous functions indispensable for maintaining life, including synthesis, metabolism and secretion. Plasma exchange and/or blood hemodialysis has been used to treat hepatic failure due to various causes, especially fulminant hepatic failure. However, the results of these treatments are not satisfactory and various types of bioartificial liver (BAL) using cultured hepatocytes have been recently developed in many institutions. Since there are numerous functions of the liver that have not yet been clarified, it is impossible to perform all these hepatic functions with a BAL system. Because of the liver's excellent regenerative ability and its great capacity in providing reserve functions, the BAL system is currently aimed at fulfilling some hepatic functions temporarily, thereby helping to ease the burden on damaged liver and overcome the peak period of hepatic failure. The only full-fledged treatment for patients with hepatic failure is liver transplantation, but the shortage of donors has become a grave problem, and the number of patients who can promptly receive a liver transplantation, especially those with acute liver failure of high urgency, is limited. For this reason, efforts are being targeted at development of an artificial liver with long-term viability in order to maintain life and to support regeneration by the liver itself, thereby serving as a bridge until a liver transplant donor can be found.

We report the current status and unresolved problems of artificial liver support systems based on our experience such as plasma exchange, blood purification and development of a hybrid bioartificial liver with reports of previous literatures.

Engineering artificial liver

Concerning engineering aspects of artificial liver, plasma exchange, blood hemodialysis and absorption-type liver support systems are currently used for patients with liver failure. Nevertheless, the results obtained with these treatments have not been satisfactory. Plasma exchange, a method in which plasma ingredients are separated by means of a plasma separating membrane from the patient's blood by plasmapheresis and a substitution fluid is administered as replacement, was reported by Lepore et al as a treatment for hepatic coma. The pore size of the plasma separating membrane is larger than that of the column used in filtration dialysis. The membrane is capable of removing substances over a broad range of molecular weights, not only substances of small or medium molecular weight, but also protein-bound substances and macromolecular substances. Moreover, because fresh frozen plasma (FFP) is used as a substitution fluid, such things as osmotic protein and coagulation factors can be supplemented. Nevertheless, it is difficult to remove substances that pool extensively in the body: for example, bilirubin or ammonia. Moreover, the volume of FFP required for one plasma exchange session is normally 3,000 ml or more, and if the procedure is continued for...
more than one day, an enormous volume would be necessary. In addition, the levels of ammonia and sodium in FFP are higher than in normal plasma, and if large volumes are administered, the electrolyte imbalance can be found. There are numerous other disadvantages to the human body, including decrease of various essential substances produced by the body or production of unnecessary substances due to FFP administration.

In blood hemodialysis, the pore size is smaller than in the column used in plasma exchange. Dialysis fluid flows through the extra-fiber space of hollow fibers, but if the flow volume increases, substances of small or medium molecular weight can be removed. It is believed that the substances causing hepatic coma in fulminant liver failure are of small or medium molecular weight. It has been reported that when dialysis fluid is perfused through the extra-fiber space of hollow fibers at a speed of 500 ml/min, an improvement of consciousness level was recognized in patients with hepatic coma. On the contrary, there is a disadvantage of inadequate removal, regardless of whether or not they are necessary for the body, based on our results from measurements of amino acids in blood and in secreted fluids before and after hemodialysis (Fig. 1).

Plasma exchange or blood hemodialysis are the major modes of artificial livers. Favorable results from treatment with engineering artificial liver have been reported in cases of hepatic failure, but the survival rates in fulminant hepatitis have remained low, at 40–60% with the acute type and 10–30% with the subacute type. In recent years, continuous hemodiafiltration (CHDF) and slow plasma exchange are mainly applied for acute liver failure. Especially, slow plasma exchange has a high ability to remove the bilirubin compared to the conventional plasma exchange. In addition, it can prevent lung or brain edema due to acute change of blood osmotic pressure, which are sometimes encountered in patients with renal failure during the treatment of conventional plasma exchange. On the contrary, some physicians report that CHDF is not useful for fulminant liver failure, because the removal rate of substances is extremely low. Therefore, its effectiveness is still controversial. In our experiments, plasma exchange and blood hemodialysis were performed on pigs with fulminant liver failure, but no salient beneficial effects could be recognized. As mentioned previously, among the potential reasons for this are the following:

1) FFP
is not necessarily good for the body, 2) continuous therapy is limited because large volumes of FFP are required, and 3) selective removal of substances is impossible.

**Biological artificial liver**

The biological artificial liver involves two processes: 1) xenogeneic extracorporeal liver perfusion, in which the patient's blood is perfused through a whole liver excised from a pig or dog, 2) hepatocyte transplantation, in which isolated human hepatocytes are injected, e.g. through the portal vein.

As treatment for liver failure, xenogeneic liver perfusion was first reported in 1958 by Otto et al\(^\text{[16]}\) and vigorous research on it was conducted in the 1960s and 1970s\(^\text{[19]}\). Over 100 cases of clinical application of the procedure have been reported to date. Ikai et al performed xenogeneic extracorporeal liver perfusion using pig liver, and confirmed that the procedure supplements liver functions and that it does not cause serious complications in primates if hemolysis can be avoided\(^\text{[19]}\). In more recent research, however, it was found that when porcine islet cells and human kidney cells are co-cultured, porcine endogenous retrovirus expression (PERV) infects the human cells\(^\text{[19]}\). On the other hand, Paradis et al reported that no gene expression of PERV was detected in the serum from 160 patients with transplanted pig cells of liver, kidney, pancreas, and skin by the RT-PCR or the immunoblot method\(^\text{[27]}\). It is evident, therefore, that a consensus on the PERV infection and risks in humans has not yet been reached and that further studies on safety are necessary.

Isolated hepatocytes can be injected throughout the human body including the liver, spleen, thyroid, testis, adipose tissue or subcutaneous tissue. Most recently, two methods have become commonplace: injection into the spleen or injection into the portal vein. In the case of injection into the spleen, because venous blood from the spleen flows into the liver, the patient's liver itself plays a role in excretion of bile produced by transplanted hepatocytes. Moreover, in the spleen parenchyma, because there is an abundance of growth factors and other cytokines, the number of hepatocytes increases, whereas transplanted hepatocytes become atrophic at other sites. It is believed that injection into the portal vein, as opposed to other anatomical sites, is more advantageous in physiological terms. By this approach, mutual reactions occur between the transplanted hepatocytes and other hepatocytes or non-interstitial cells, and this could enhance secretion of bile into the bile duct. However, reports of hepatocyte transplantation in actual clinical applications are limited\(^\text{[28,19]}\). Moreover, the number of transplanted hepatocytes is small and reports on post-transplant results are insufficient\(^\text{[26,21]}\).

The reason is that the opportunities for providing hepatocytes for transplant are scant, because whole liver is used in routine liver transplantation. In the future, therefore, technology must be established for isolation and cultivation of hepatocytes or stem cells in sufficient quantities as required for hepatocyte transplantation from portions of liver from brain-dead donors.

**Hybrid bioartificial liver**

Since it became possible to isolate hepatocytes with high activity by the collagenase perfusion method developed by Berry and Friend in the 1960s\(^\text{[23]}\), vigorous efforts have been made to develop a hybrid bioartificial liver using isolated hepatocytes. Subsequently, a monolayer culture of hepatocytes was established, which is currently in wide use, but the period of maintenance is limited. On the other hand, in recent years, three-dimensional culture methods, including spheroid culture, have allowed advanced functions to be maintained for several weeks. This method is expected to serve as a promising avenue for development of the hybrid bioartificial liver\(^\text{[23]}\).

The outline of the hybrid bioartificial liver is shown in Fig. 2. Plasma is isolated from patient's blood in a plasma separation column, then allowed to come in contact with cells filled inside a bioreactor. The purpose of these cells is to metabolize and excrete unnecessary substances from plasma of the patients with liver failure, and to synthesize and supplement coagulation factors and other essential proteins. There are several problems with the structure of the bioreactor, the cells filling the bioreactor and the structure of the device that includes the plasma separating membrane. Because it is impossible to obtain large volumes of normal human hepatocytes, porcine cells are usually used clinically for the hybrid artificial liver in Europe and North America. For instance, Hui et al cultured first-generation of iso-

![Fig. 2 Basic diagram of the hybrid bioartificial liver](image-url)
lated porcine hepatocytes on collagen-coated dextran microcarriers, and put them into the extra-fiber space of hollow fibers in order to produce an artificial liver. Then, the BAL system was used continuously for 5 to 7 hours per day, yielding favorable results as a bridge to transplantation in patients with fulminant liver failure. Sussman et al. performed the cloning of C3A cells from established hepatocytes originating from human hepatoblastoma, and used them as a bioreactor cell. In their system, volumes of hepatocytes weighing 30 g were cultivated for 3 to 4 weeks until they reached 200 g, and then were used in actual clinical therapy. They were applied in 11 cases, including severe drug-induced liver failure or fulminant liver failure, in 4 of which cases the patients could undergo liver transplantation, while in another 2 cases, the patients recovered to the point that an artificial liver was no longer necessary. In Japan, a group in Kyushu University has added a spheroid of isolated porcine hepatocytes into polyurethane foam for development of the BAL system, which maintains a sufficient volume of hepatocytes to serve as a liver substitute in patients with fulminant liver failure. Animal experiments were then conducted using pigs suffering from warm ischemic liver failure, and favorable results such as prolongation of survival period, and maintenance of blood glucose and ammonia level were obtained. Currently, the Kyushu University group has submitted a proposal to the ethics committee for clinical application of this system.

At present, the main purpose of the BAL system is as a bridge to liver transplantation, and almost all cases of clinical application in Europe and North America belong to this category. However, in these cases the BAL system could not be used for long periods. In Japan, on the other hand, there have only been 10 cases of liver transplantation from brain-dead donors thus far, and the shortage of donors is a serious problem. Therefore, interest in the clinical applications of the hybrid bioartificial liver extends not only to its use as a bridge to liver transplantation but also to its long-term, life-saving potential, independent of liver transplantation (Fig. 3). In this respect, one mode of research on the artificial liver has been an attempt to establish immortalized human hepatocytes. In various studies, HepG2 strain originating from hepatoblastoma is most widely used as a substitute for human hepatocytes. Also widely used in Japan are HuH-7 and huH-1, established from cancerous hepatocytes. In our study, we used GS-HepG2 cells, in which the genes of glutamine synthesizing enzyme were introduced into the HepG2 strain, in order to elevate ammonia metabolizing capacity. The Cygnus (Meiji Milk Co. Ltd, Tokyo, Japan) was used as a bioreactor, which traditionally has been used as a circulatory type incubator. As shown in Fig. 4, GS-HepG2 cells adhered to glass fibers wrapped in a tree-ring-like shape. The most characteristic feature of this bioreactor is that in addition to the circuit connected to the patient, there is another circuit through which cultured fluid is perfused continuously in order to maintain the cells. In the conventional bioreactor, cultured cells are maintained by the patient's plasma; while the fluid for culture and maintenance of bioreactor cells is perfused through a separate circuit, resulting in a more favorable environment for these cells. As a result, GS-HepG2 cells were cultured in the Cygnus for over 100 days, and the ammonia metabolizing capacity was maintained at one-sixth of the initial capacity of the initially isolated porcine hepatocytes. In addition, the cell count increased from $5 \times 10^5$ to $4 \times 10^6$. During this period, this system could be used four times repeatedly in in-vivo experiments with pigs (Fig. 5). In order to evaluate the clinical effects of this bioreactor, an acute liver failure was introduced by pigs, and perfusion experiments were conducted with the
following groups as controls: non-treatment group (NT group), group using cell-free reactor (CF group), plasma exchange plus blood hemodialysis group (PE + CHDF group). Then, comparisons were made with the wild type HepG2 group (w-HepG2 group) and the GS-HepG2 group\(^{20,31}\). In the GS-HepG2 group, the 50% average survival period was about 16 hours, whereas it was no more than 10 hours in the NT group, the CF group, the PE + CHDF group and the w-HepG2 group. The survival period was significantly prolonged in the GS-HepG2 group (Fig. 6). In addition, a tendency to improve was also noted in the various coagulant factors in the GS-HepG2 group (Fig. 7). Approximately 44% of the control group animals died of cerebral hernia with rapid decrease of intracranial pressure immediately after rapid increase, while the percentage was low (12.5%) in the GS-HepG2 group and in the w-HepG2 group with statistical significance (Fig. 8). This result suggests that HepG2 cells originating from hepatoblastoma might inhibit an increase of intracranial pressure by some mechanism. In conclusion, unlike in the conventional system, bioreactor cells can be cultured for long periods, and viability can be maintained in our BAL system. On the other hand, the problem with the BAL system using immortalized hepatocytes may be associated with the risk of tumor formation. Since it is impossible to produce a complete liver substitute with hepatocytes alone, non-hepatic cells such as sinusoidal epithelial cells should also be co-cultured.

At present, liver transplantation is the most effective therapy for acute or chronic liver failure, but in Japan...
there are many patients who cannot be saved because of a persistent shortage of donors. Plasma exchange or blood hemodialysis is found to be effective in only a limited number of patients. To ameliorate this situation, ceaseless efforts must be directed into developing a hybrid artificial liver that can be used for long periods.

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References

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人工補助肝臓の最新の進歩と問題点

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種々の原因による肝不全、特に原発性肝不全を治療するために、血漿交換、血液ろ過透析などが行われてきたが、これらの治療成績は満足のいくものではないため、近年、培養肝細胞を用いた種々の人工肝の作成が行われている。人工肝の目的は、一時的に肝機能の一時的代謝と、肝不全の急期を乗り切ることであり、肝不全患者に対する唯一の根本的な治療は肝移植であるが、ドナー不足が深刻な問題である。このため、肝移植のドナーが現れるまでの橋渡し療法として、生命維持や自己肝臓の再生のためにも、長期にわたって viability を有する人工肝の開発が期待されている。

我々の人工肝の研究では、HepG2 株にグルタミン合成酵素遺伝子を導入した GS-HepG2 細胞を使用した。また、バイオリアクターとして、回流式培養装置として使用されている Cygnus を使用した。従来のバイオリアクターがその構造上、患者の血漿でバイオリアクター内肝細胞を維持するのに対し、このシステムでは、バイオリアクター細胞を培養維持するための培養液を別に通路で灌流することによって、細胞に対し好環境を保つことが可能である。この結果、GS-HepG2 細胞を 100 日間以上培養でき、アンモニア代謝能も、初代分離プタ肝細胞の約 1/6 の機能を保持していた。また、細胞数は、5 × 10^11 から 4 × 10^12 まで増殖した。この間、in vivo 実験に 4 回反復使用することができ、さらに、このバイオリアクターの臨床効果を評価する目的で、プタを用いた急性肝不全モデルにおける灌流実験を行い、長期の細胞培養および viability の維持が可能であることが示された。不凍化肝細胞株を用いた BAL システムの問題点としては、腫瘍形成の危険性があることである。また、完全な代用肝を作成するためには、肝細胞 (hepatocyte) のみでは不可能であり、類細胞内皮細胞 (sinusoidal epithelial cell) などの非実質細胞 (non-hepatic cells) の導入が不可欠であり、今後の検討が期待される。

〈Key words〉肝補助療法、人工肝臓、肝不全