3.3. 数値流体解析値と動物実験から導出したガス交換性能の比較

充填体モデルを用いた数値流体解析手法による結果と急性動物実験による結果を比較するために、それぞれの結果から導いた酸素移動量を図7、炭酸ガス移動量を図8に示した。

まず、参照人工肺において中空糸膜と血液主流方向に垂直な方向の血液偏流はないと仮定して、人工肺局所部分となる数値解析結果から、ガス交換性能を推定した。血流量Q=1 L/minにおける解析酸素移動量は、V/Q=1で71.1 mL/min、V/Q=3で71.1 mL/min、解析炭酸ガス移動量はV/Q=1で80.0 mL/min、V/Q=3で83.7 mL/minであった。血流量Q=3 L/minにおける解析酸素移動量は、V/Q=1で198.7 mL/min、解析炭酸ガス移動量はV/Q=1で179.1 mL/minであった。

これらに対して、急性動物実験結果から導出した血流量Q=1 L/minにおける酸素移動量はV/Q=1で64.1 mL/min、V/Q=3で58.3 mL/min、炭酸ガス移動量はV/Q=1で117.7 mL/min、V/Q=3で136.3 mL/minとなっていた。血流量Q=3 L/minにおける酸素移動量は、V/Q=1で138.4 mL/min、炭酸ガス移動量はV/Q=1で191.0 mL/minであった。

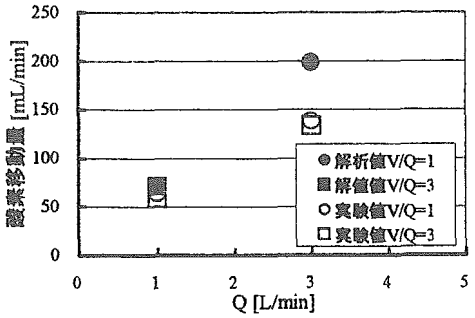

図7 人工肺ガス流入側部分における酸素移動量の解析結果より推定した値とex-vivo実験より求めた値の比較（Q=1、3L/min、V/Q=1、3）

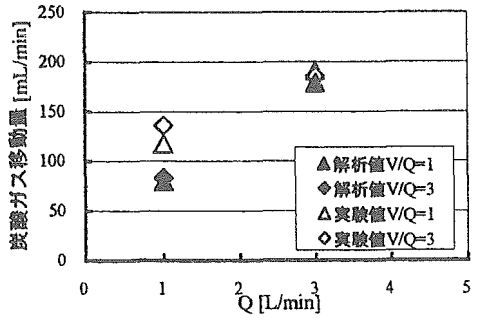

図8 人工肺ガス流入側部分における炭酸ガス移動量の解析結果より推定した値とex-vivo実験より求めた値の比較（Q=1、3L/min、V/Q=1、3）

4. 考察

4.1. 充填体モデルによる血流方向濃度分布

全ての血液層濃度分布において、血流の進行に伴い濃度が一定値に漸近する傾向が見られたのは、酸素および炭酸ガス移動にともなって、ガス層と血液層の濃度較差が減少するにつれて、移動速度勾配が平衡状態に近付くためであると考えられた。しかし、今回の解析対象となるモデルは中空糸束のガス流入側端部に相当し、ガス層の酸素および炭酸ガス濃度はそれぞれ初期流入条件に限りなく近く維持されるため、血液層の酸素および炭酸ガスもそれぞれ713 mmHg、0 mmHgに漸近すると考えられるにもかかわらず，それぞれの濃度変化は713 mmHg、0 mmHgより手前の値に漸近する傾向が見られた。この問題に関しては、今後許容誤差や解析手法に関して詳細な検討を要するものと考えられる。

血流量の増加に対し、流出部の酸素濃度および炭酸ガス濃度は明らかに変化量が減少していた。これは、濃度較差により移動する酸素と炭酸ガスの血液中の拡散移動に対し、血流量増加にともなう対流移動が支配的に効いてくるためと考えられた。

4.2. 数値流体解析値と血液実験値から導出したガス移動量による比較

酸素移動量における比較では、血流量Q=1 L/minにて実測値に対する解析値が実用上問題ないと考えられる範囲に収まったが、血流量Q=3 L/minに増加することにより、実験値に対する解析値は＋44%の高値を示した。炭酸ガス移動量における比較では、血流量Q=3 L/minにて実験値に対する解析値が実用上問題ないと考えられる範囲（−6%以下）に収まったが、血流量Q=1 L/minに減少することにより、実験値に対す

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Thoughts and Progress

The Bioreactor With CYP3A4- and Glutamine Synthetase-Introduced HepG2 Cells: Treatment of Hepatic Failure Dog With Diazepam Overdosage

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Abstract: A novel recombinant human hepatic cell line, CYP3A4- and glutamine synthetase (GS, an enzyme which converts ammonium ion and glutamate to glutamine)-introduced HepG2 (HepG2-GS-CYP3A4), was established. Its usefulness in a large-scale culture with a circulatory bioreactor in vitro and in dog models of ischemic hepatic failure with acute diazepam (DZP, a substrate of CYP3A4) overdosage was further examined. HepG2-GS-CYP3A4 expressed about 9 times larger amounts of CYP3A4 protein than a control. After incubation with HepG2-GS-3A4 cells in a circulatory bioreactor for 24 h, ammonia and DZP concentrations in the culture medium significantly decreased by about 40%. Furthermore, this system improved the survival time and decreased serum concentrations of DZP, ammonia, and transaminase in dogs with ischemic hepatic failure plus acute DZP overdosage. The mean survival time with bioreactor with HepG2-GS-3A4 was 42.7 ± 3.6 h, which was significantly longer than that without reactor, with reactor (no cells), and with HepG2-GS (23.4 ± 2.8, 22.1 ± 2.4, and 31 ± 3.7 h, respectively). Therefore, it is concluded that this bioartificial liver could be a good tool for the treatment of dogs with hepatic failure and that it could potentially be a bridging procedure to liver transplantation. **Key Words:** Bioartificial liver—Diazepam intoxication—Hepatic failure—CYP3A4—HepG2—Dog.

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Hepatic failure leads to the accumulation of multiple water-insoluble toxins, such as unconjugated bilirubin and endogenous benzodiazepines (1). Especially, endogenous diazepam (DZP) and nordiazepam (NDZP) are considered to have a pivotal role in the pathogenesis of hepatic encephalopathy (2,3). Elimination half-life of DZP is five times longer in patients with liver cirrhosis than control subjects because the activity of cytochrome P450 3A4, a major metabolizing enzyme for this compound, is impaired. Therefore, providing metabolizing ability for DZP and other hepatic toxins is important for the treatment of hepatic failure.

A bioartficial liver (BAL) support system is expected to be a new tool for terminal-stage liver failure and for bridge-to-transplantation use (4). Enosawa et al. developed recombinant HepG2 cells with the introduction of glutamine synthetase (GS), an enzyme which converts ammonium ion and glutamate to glutamine, and applied them to a novel type of circulatory cell culture system (5,6). They reported significant removal of ammonia by this system in a hepatic failure model, but they could not achieve improvement in survival time in this model. These results indicate that some toxins other than ammonia are involved in hepatic failure, and intrinsic benzodiazepine might be a candidate.

The objectives of this study were: (i) to establish HepG2-GS-3A4 cells with the introduction of both GS and CYP3A4 genes; (ii) to assess the usefulness of HepG2-GS-3A4 cells in a BAL device in vitro; and (iii) to evaluate the system as a treatment modality for a canine model of acute hepatic failure combined with a DZP overdose.

METHODS

Cells and Western blot analysis for human cytochrome P450 3A4

HepG2 cells were obtained from the RIKEN cell bank (Tsukuba, Japan). HepG2-GS-3A4, a cell transfected with both the hamster GS gene and the human cytochrome p450 3A4 gene was established by lipofection of pBudCE4 (Invitrogen, Carlsbad, CA, U.S.A.) after ligation of both genes. A positive clone was selected by an index of resistant against zeocin. HepG2-GS-3A4 cells were cultured in DMEM/F-12: RPMI (1 : 1), with 10% FCS + zeocin. Twenty micro-

grams of protein was resolved by 7% SDS-PAGE electrophoresis, and immunoblotted with human CYP3A4 antibody (WB human CYP3A4 kit, BDGenetest, MA, U.S.A.).

Long-term culture in circulatory flow bioreactor for bioartficial liver (BAL) and treatment of acute hepatic failure with DZP overdosage

The circulatory flow bioreactor originally developed for culturing adhesive cells (US Patent no. 5270207) (5), was used as a bioartficial liver (6). During the culture, fresh culture medium, air, and CO_2 were continuously supplied to the vessel and the temperature was maintained at 37°C. Evaluation of the metabolizing capacity was performed at 20 days after inocculation of 1×10^8 cells. To evaluate metabolizing activity of DZP and ammonia, these compounds (2.5 mm and 30 μg/mL, respectively) were added to the fresh medium and the old medium flowing out from the reactor was sampled.

Beagle dogs (10–20 kg, $n = 23$) were anesthetized with pentobarbital (1 mg/kg, i.v.). The procedure of Abouna (7) was partly modified to establish an ischemic hepatic failure model with an end-to-side portal–vena cava shunt and ligation of hepatic arteries. One and half hours after operation, DZP was intravenously infused to each dog at a dose of 3 mg/kg. Blood samples were taken at scheduled times to check the level of ammonia, DZP, its metabolites, and aminotransferases. Dopamine (30 mg/kg/h at maximum dose) was infused to maintain arterial blood pressure. Termination of heartbeat was regarded as the end of survival.

Treatment with the bioartificial liver was performed just after the infusion of DZP. During the operation, culture medium was completely replaced with artificial solution for hemoperfusion and thereafter changed to 550 mL of dog plasma (6). A catheter was introduced into the femoral artery for drawing blood at 30–50 mL/min. The blood flowed into a plasma separator column (OP-02 W, Asahi Medical, Tokyo, Japan) and the separated plasma flowed into the BAL at a rate of 150–250 mL/h.

The concentrations of ammonia and transaminase were measured by modified Berthelot reaction and Reitman and Frankel's method (8) with autoanalyzer, respectively. Concentrations of DZP were measured by high-performance liquid chromatography (9). Values were expressed as the mean ± S.E. ANOVA and *t*-test were used as appropriate for statistical comparison. Survival rate for each treatment was compared by logrank analysis. $P < 0.05$ was regarded as significant.

RESULTS

Confirmation of human cytochrome P450 3A4 in HepG2-GS-3A4 cells and metabolism of ammonia and DZP in BAL system

The HepG2-GS-3A4 cells had about nine times higher amounts of CYP3A4 protein than HepG2 in Western blot analysis. Densitometric values were 0.18 ± 0.02 and 0.02 ± 0.01 units for HepG2-GS-3A4 and HepG2, respectively.

Concentrations of ammonia and DZP after incubation with HepG2-GS-3A4 cells in the bioreactor for 24 h are shown in Table 1. Ammonia and DZP concentrations significantly decreased with HepG2-GS-3A4 cells. HepG2-GS showed almost the same ammonia metabolism capacity as HepG2-GS-3A4 and caused nearly no DZP reduction. The initial removal rate of ammonia by the reactor with HepG2-GS-3A4 cells was about 0.4 nmol/min/10^6 cells. The amount of DZP metabolized by HepG2-GS-3A4 in the reactor, which was calculated from differences of the area under concentration-time curves between inlet and outlet of the medium and subtraction of the value obtained from the experiments, was significantly higher in HepG2-GS-3A4 (Table 1).

Treatment of acute hepatic failure with DZP loading by HepG2-GS-3A4 in the bioreactor

Treatment using the bioreactor with HepG2-GS-3A4 significantly prolonged survival time to 42.7 ± 3.6 h (Fig. 1). Treatment with HepG2-GS also prolonged mean survival time to 31 ± 3.7 h, but it did not reach statistical significance ($P = 0.08$). The time course of GPT, ammonia, and DZP are shown in Fig. 2. GPT and ammonia concentrations increased

TABLE 1. *Metabolism of ammonia and DZP by HepG2-GS-3A4 in the bioreactor in vitro*

	Concentration 24 h after incubation		Metabolized diazepam amount for 24 h (μg/hL)
	Ammonia (mM)	Diazepam (μg/mL)	
HepG2-GS-3A4 ($n = 4$)	1.5 ± 0.2**	19 ± 1*,**	74 ± 9*
HepG2-GS ($n = 3$)	2.0 ± 0.2**	23 ± 1	20 ± 10
Without cells ($n = 3$)	2.4 ± 0.2	24 ± 2	—

*$P < 0.05$ vs. HepG2-GS, **$P < 0.05$ vs. without cell.

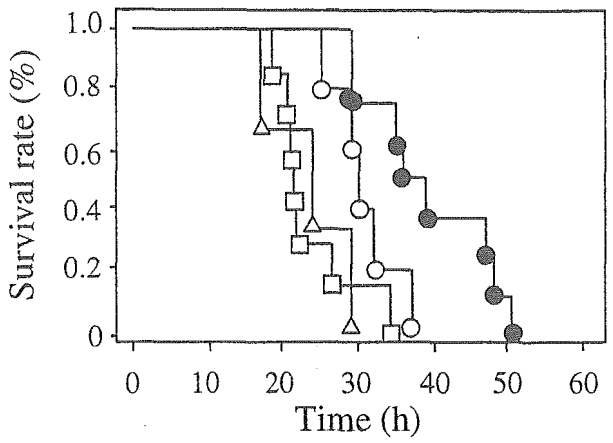

FIG. 1. Cumulative survival period for hepatic failure with DZP loading treated by a bioreactor with HepG2-GS-3A4. ● HepG2-GS-3A4 ($n = 8$); ○ HepG2-GS ($n = 5$); □ without cells ($n = 7$); △ without bioreactor (hepatic failure alone, $n = 3$).

with time in all groups, however, the increase at 24 h was less in the HepG2-GS-3A4 group for GPT and in both HepG2-GS-3A4 and HepG2-GS groups for ammonia. The reduction of DZP concentration was significantly more rapid in HepG2-GS-3A4 than other groups.

DISCUSSION

In this study, we clearly demonstrated the ability of HepG2-GS-3A4 cells in the bioreactor to metabolize DZP and ammonia. Thus, this is the first effective system that removes both DZP and ammonia, which accumulate in hepatic failure. In addition, this is the first report that shows the effectiveness of a system for removal of these molecules from circulating blood in an animal model. Although the clearance of ammonia and DZP was significant in our report, it still remains lower than normal hepatocytes. However, porcine hepatocytes have many disadvantages in a BAL system, such as xenoantigenicity, xenozoonosis, laborious handling, and difficulties in storage, although they have high capacity and multiple functions (6). As the HepG2-GS-3A4 cell line does not have any of these disadvantages, it is still a good candidate for the treatment of human hepatic failure.

When the acute hepatic failure dogs with DZP overdosage were treated using the bioreactor with HepG2-GS-3A4, DZP and ammonia concentrations were significantly reduced, and subsequently the survival period was significantly increased. This is the most important observation in this study. Similarly to the results of Enosawa, the reactor with HepG2-GS could decrease the plasma concentration of ammonia, but the survival period did not increase significantly (6). Although GPT was obviously lower during the treatment using the reactor with HepG2-GS-3A4, ammonia concentration with HepG2-GS-CYP3A4 did not differ from that of HepG2-GS. This observation suggests that toxins other than ammonia, which are metabolized by CYP3A4 such as endogenous benzodiazepins, are important for the mortality rate in hepatic failure. Although we did not measure intracranial pressure, the removal of intrinsic/extrinsic benzodiazepeins might reduce the pressure and prolong the survival. Alternatively, this BAL might not only help the dysfunctional liver to detoxify

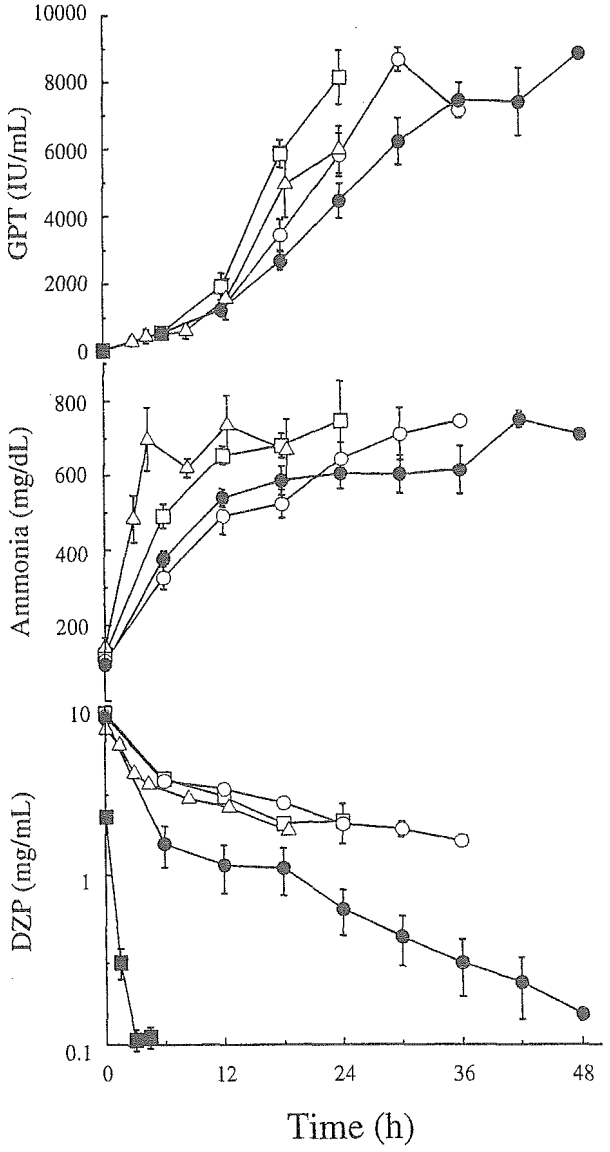

FIG. 2. Time course of GPT (upper panel), ammonia (middle panel), and DZP (lower panel) during treatment. ● HepG2-GS-3A4 ($n = 8$); ○ HepG2-GS ($n = 5$); □ without cells ($n = 7$); △ without bioreactor (hepatic failure alone, $n = 3$); ■ healthy dog ($n = 3$).

toxins, but it might also guard against destruction of hepatocytes.

In conclusion, the present study suggests that this system with HepG2-GS-3A4 can be potent for the treatment of dogs with hepatic failure when ammonia and endogenous benzodiazipines are high. This system might be useful as a bridge to liver transplantation, although more modification is needed to use it in clinical practice.

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Construction and Evaluation of Drug-Metabolizing Cell Line for Bioartificial Liver Support System

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Focusing on drug metabolism in liver, we constructed and evaluated a drug-metabolizing bioartificial liver (BAL) support system. In a previous study, we constructed ammonia-metabolizing CHO and hepatoma-derived HepG2 cell lines by recombination of the glutamine synthetase (GS) gene. For further mimicking of liver metabolism, the human hepatoma-derived cell line HepG2 was transformed by the pBudCE-GS-CYP3A4 vector, which contains GS and drug-metabolizing CYP 3A4 genes. The constructed GS-3A4-HepG2 cell line showed 3A4 activity higher than that of human primary hepatocytes. The drug-metabolizing activity of BAL (BAL clearance) was evaluated using this cell line. The estimated clearance was higher than that of the human hepatocyte system.

Introduction

The liver is capable of regenerating itself after acute injury but cannot support human functions during the regeneration process in cases of serious injury. Bioartificial liver (BAL) support systems composed of artificial materials and living cells were developed as "bridging" devices for use during liver regeneration to support patients with hepatic failure (*1*, *2*). Many researchers developed BAL systems using xenogenic primary hepatocytes, i.e., porcine, dog, rat, etc. Xenogenic primary hepatocytes can show high liver functions in vitro, and xenogenic BAL systems are extremely effective in short-term use (a few days) but are not suitable for long-term recycling use (more than 1 month) because of the short lifespan of the primary hepatocytes and a tendency to lose the liver functions during in vitro cultivation (*1*, *3*). Furthermore, because of the risk of zoonosis, use of primary hepatocytes has been an ongoing debate (*1*). To overcome this disadvantage, recently various 3D cultivation methods, i.e., collagen sandwich cultivation (*4*), modular extracorporeal liver support (MELS) (*2*), and multicapillary polyether rigid-type polyurethane foam (MC-PUF)/hepatocyte spheroid packed-bed module (*5*), were developed. These devises could attain long-term usage of primary hepatocyte more than 1 week. On the other hand, hepatic cell lines do not pose the risk of zoonosis and are suitable for long-term recycling use; however, most of the liver functions are missing in them. The safety aspects of using a hepatoma cell line or genetically altered cell line still need to be addressed. Only one experimental program is clinically tested, a BAL device using HepG2-derived C3A cell line (*6*). In previous studies, we constructed an ammonia-metabolizing human HepG2 cell line (GS-HepG2) and successfully supported a pig with liver failure, using a BAL system with a circulatory flow bioreactor (*7*–*11*). This constructed GS-HepG2 cell line could attain long-term recycling usage (more than 6 months) (*7*–*11*). However, drug-metabolizing activity, one of the most important functions of liver detoxification, remained at a very low level in GS-HepG2. High levels of drug-metabolizing activity could be maintained for more than 1 week by xenogenic primary hepatocytes if 3D cultivation were used (*12*). However, xenogenic primary hepatocytes are essentially different from human hepatocytes. Drug metabolism by the P450 enzyme is different between humans and other animals because of the differences of CYP subfamily species (*13*). Between these families, the substrate, inhibitor, inducer, and products are varied. In addition, the P450 activity of primary hepatocytes decreases rapidly during in vitro cultivation. For example, after cultivation for 24 h, P450 3A4 activity decreased to less than 50% of in vivo activity (*14*). In this study, we constructed an ammonia- and drug-metabolizing HepG2 cell line (GS-3A4-HepG2) for a BAL system and considered evaluation methods for the drug-metabolizing BAL system using the GS-3A4-HepG2 cells.

Materials and Methods

Cell Line and Culture Medium. The cell lines employed in the experiments were the CHO-K1 (RCB 0285), HepG2 (RCB0459), GS-CHO (*7*), and GS-HepG2 (RCB 1680) (*7*) cell lines. The tissue culture medium was an RDF (HO) medium containing glucose (2.58 g/L), glutamine (333 mg/L), and 10% dialyzed fetal bovine serum (GIBCO). The RDF (HO) medium is a 2:1:1 mixture of RPMI1640, Dulbecco's modified Eagle's MEM (DMEM), and Ham's F12 without glucose and glutamine (*15*, *16*). The selection medium contained glutamic acid (336 mg/L) and NH_4Cl (122 mg/L) instead of glutamine, and Zeocin (Invitrogen R250-01) (200 μg/mL for CHO-

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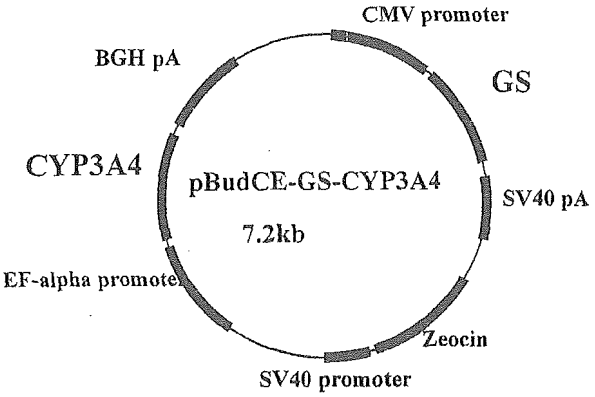

Figure1. pBUdCE-GS-CYP3A4 vector

Figure 1. pBUdCE-GS-CYP3A4 vector.

K1 and 300 μg/mL for HepG2) for selection of the recombinant.

Construction of Expression Vector for CYP3A4 and Glutamine Synthetase. Using oligonucleotides A, 5′-TTTTTTGCGGCCGCGTGATGGCTCTCATCCCAG-ACTTGG-3′ (upstream primer containing a *Not* I site), and B, 5′-GGGGGGAGATCTATTCAGGCTCCACTTA-CGGTGCCATC-3′ (downstream primer containing a *Bgl* II site), we obtained a full-length CYP3A4 cDNA by using pLNCX-CYP3A4 (kindly provided as a gift by Dr. Els. M. De Grone (*17*)) as a PCR template. Oligosaccharide A contained the initiation Kozak sequence (*18*) for protein biosynthesis in an optimal sequence context, A/G NN ATG G. The amplified product was digested with Not *I*/Bgl *II* and ligated into the Not *I*/Bgl *II* sites between the EF-1 alpha promoter and BGH poly A of pBudCE4 (Invitrogen V532−20). Using oligonucleotides C, 5′-AAAAAAAAGCTTACCATGGCCACCTCAGCAAGTT-CCC-3′ (upstream primer containing a *Hind* III site), and D, 5′-CCCCCCGGATCCAATTAGTTTTTGTATTGGAA-GGGCTC-3′ (downstream primer containing a *Bam HI* site), we obtained a full-length glutamine synthetase (*gs*) cDNA by using pBK-CMV-GS (*7*) as a PCR template. Oligosaccharide C also contained the initiation Kozak sequence. The amplified product was digested with *Hind* III/*Bam HI* and ligated into the *Hind* III/*Bam HI* sites between the CMV promoter and SV40 poly A of pBudCE4. The nucleotide sequence *CYP3A4* and *gs* were determined and found to be correct. The constructed pBUdCE-GS-CYP3A4 vector contains *CYP3A4* and *gs* genes (Figure 1). The CHO-K1, HepG2, and GS-HepG2 cells were transformed by the pBUdCE-GS-CYP3A4 vector using the lipofection method (TransIT transfection reagent kit, TAKARA).

Evaluation of Catalytic Activity of P450 3A4, Glutamine Synthetase, and Ammonia-Metabolizing Activity. Cells were inoculated at 1×10^7 cells per 100-mm culture dish and incubated for 16 h at 37 °C with 5% CO_2. The culture medium was replaced with 10 mL of fresh medium, and testosterone (100 mM stock in methanol) was added directly into the cultured medium until the final concentration was 100 μM. In cases where the Michaelis−Menten parameter was determined, the testosterone concentration ranged from 25 to 400 μM. After 2 h of incubation at 37 °C with 5% CO_2, a 2-mL aliquot of the medium was removed for a HPLC assay (*14*). The 6β-testosterone hydroxylase activity of CYP3A4 was measured by reversed-phase HPLC. Androstenedione was used as an internal standard for the HPLC assay. The cells were harvested by trypsinization and lysed by sonication. In a P450 3A4 induction experiment, cells were maintained for 6 days using a serum-free medium with rifampicin. After cultivation for 6 days, the 6β-testosterone hydroxylase activity was evaluated. The protein concentration was determined using a BCA protein assay reagent kit (Pierce 23225). Ammonia-metabolizing activity was evaluated in a T-flask culture (medium volume 90 mL) as previously described (*7*). Briefly, during more than 50 h cultivation, we measured the cell concentration and ammonia concentration using photomicrography and enzyme reaction kit, respectively (*15*, *16*). Photo micrographic measurement of cell concentration was correlated to measurement by hemacytometer using the trypan blue dye exclusion method. Based on the time course of cell and ammonia concentrations, we calculated the specific ammonia consumption rate (mol/cell/h) using the integral method (*15*). In the case of gene amplification of glutamine synthetase, the recombinant cells were selected under different concentrations of the glutamine synthetase inhibitor, methionine sulfoximine (MSX). Glutamine synthetase activity in crude extracts of the cells was determined according to the γ-glutamyl transfer reaction (*19*).

Table 1. Testosterone 6β Hydroxylation by Cultured Cells

	pmol/min/mg of total cellular protein	n =
GS-3A4-HepG2	490 ± 10	7
GS-3A4-HepG2 (after 80-day passage culture)	426	1
GS-3A4-CHO	21 ± 1.3	7
GS-3A4-GS-CHO	27 ± 1.5	4
HepG2	0.6 ± 0.05	5
GS-HepG2	0.7	1
HepG2 (induced by 100 μM rifampicin)	2.3[a]	1
GS-HepG2 (induced by 50 μM rifampicin)	1.1[a]	1
human primary hepatocyte (96 h after isolation)[b,c]	11	
human primary hepatocyte (24 h after isolation)[b,d]	253 ± 110	

[a] Maximum value between 10, 50, 100, and 200 μM rifampicin induction. [b] Testosterone 6β hydroxylase activity at testosterone concentration of 250 μM. [c] Reference 19. [d] Reference 20.

Results and Discussion

Evaluation of P450 3A4 and Ammonia-Metabolizing Activity of Cultured Cells. To construct the drug- and ammonia-metabolizing cell lines, CHO-K1, HepG2, GS-CHO, and GS-HepG2 cell lines were transformed by the pBUdCE-GS-CYP3A4 vector containing the *gs* and *CYP3A4* genes. After the transformation, the cells became confluent within 2 or 3 days while an RDF (HO) medium without Zeocin was used. After that, cells were transferred into the selection medium containing Zeocin. After a selection period of more than 2 months, we selected the GS-3A4-CHO, GS-3A4-GS-CHO, and GS-3A4-HepG2 cell lines. We were not able to select any transformants from GS-HepG2. The 6β-testosterone hydroxylase activity of the constructed cell lines was investigated (Table 1). The constructed GS-3A4-CHO cell line showed 21 pmol/min/mg of total cellular protein activity of testosterone 6β hydroxylation. This activity was 35 times that of the HepG2 or GS-HepG2 cell lines. GS-3A4-GS-CHO, which was a transformant of the pBUdCE-GS-CYP3A4 vector into GS-CHO, showed 27 pmol/min/mg of total cellular protein activity, similar to that of GS-3A4-CHO. The endogenous P450 3A4 activity

of HepG2 might be increased by induction. Rifampicin was one of the strongest inducers for P450 3A4. Therefore, we attempted to increase the P450 3A4 activity of the HepG2 and GS-HepG2 cell lines by adding the inducer rifampicin. The HepG2 and GS-HepG2 cell lines increased testosterone 6β hydroxylation activity to 330% and 160%, respectively. However, this activity was not comparable to that of GS-3A4-CHO.

In the GS-3A4-HepG2 cell line, the activity of testosterone 6β hydroxylation was 490 pmol/min/mg of total cellular protein. Testosterone 6β hydroxylation was mainly catalyzed by the P450 3A4 enzyme. The cause of this high level of 6β hydroxylation activity should be expression of exogenous P450 3A4. In a comparison between the GS-3A4-CHO and GS-3A4-HepG2 cell lines, the activity of GS-3A4-HepG2 was about 20 times that of GS-3A4-CHO. P450 3A4 is a mixed-function oxidase of the cytochrome b type. An adequate NADPH supply is necessary for P450 3A4 activity. P450 reductase was strongly expressed in liver-derived cell lines because of the originally supporting high activity levels of various P450 enzymes. GS-3A4-HepG2 might have a high activity level in the P450 3A4 NADPH recycling system.

It was difficult to directly compare human primary hepatocytes and the constructed cell lines because the P450 functionality of primary hepatocytes tends to decrease during cultivation. Williams et al. evaluated testosterone 6β hydroxylation and its induced activity of human hepatocytes from a human transplant donor after cultivation for 96 h (*20*). Similarly, Donato et al. evaluated human hepatocytes from surgical human liver biopsies after cultivation for 24 h (*21*). The data from both studies was obtained under a testosterone concentration of 250 μM with monolayer culture (Table 1). Wortelboer et al. estimated that the P450 enzyme activity of rat hepatocytes decreased 50% during 24 h of cultivation after isolation (*14*). Therefore, the testosterone 6β hydroxylation activity of human primary hepatocytes was estimated as ranging from about 300 to 700 pmol/min/mg of total cellular protein. Also, there are numerous references that report the P450 metabolism data based on "microsomal" protein contents. Pearce et al. reported that the microsomal testosterone 6β hydroxylation activity was 3,500−3,700 pmol/min/mg of microsomal protein (*22*). Geraldine et al. also reported 500−13,000 pmol/min/mg of microsomal protein varied with induction (*23*). Hepatic microsomal protein content was about 12.5% of total cellular protein (*24*). It was estimated that these microsomal activities corresponded to from about 62.5 to 1,625 pmol/min/mg of total cellular protein. The testosterone 6β hydroxylation activity of GS-3A4-HepG2 was quite comparable in order of magnitude to that of human liver. To evaluate the stability of the constructed GS-3A4-HepG2, cells were maintained under a passage culture of more than 80 days with selection medium containing Zeocin. After a passage culture of 80 days, GS-3A4-HepG2 had the same activity as in the initial phase (Table 1). GS-3A4-HepG2 could maintain the activity after the passage of 1 year (data not shown). This long-term stability could be attained under the selection medium. In case of BAL treatment for patient, we should remove such antibiotics during operation. Further study for long-term stability without antibiotics is necessary for evaluation. GS-3A4-HepG2 is a highly functional human liver cell line with long-term stability, and it seems to be a suitable cell line for BAL systems. It was well-known that the 3D cultivation could enhance and maintain the liver functions of primary hepatocyte. In case of cell line, Fukuda et al. reported that 3D MC-PUF culture enhanced the ammonia-metabolizing activity of the HepG2 cell line (*25*). We evaluated the liver function of the GS-3A4-HepG2 cell line using a monolayer culture. It could be expected that these 3D cultivations would enhance the liver function of GS-3A4-HepG2. Of especial note is that P450 3A4 is the most widely expressed drug-metabolizing enzyme in humans and shares 28.8% of total P450 enzyme (*26*). Moreover, about half of drugs were metabolized by P450 3A4 (*27*). Drug-induced hepatocellular injury is one of the most serious and common forms of hepatopathy (*28*, *29*). The GS-3A4-HepG2 cell line is expected for supporting BAL systems having a particular focus on drug-induced hepatocellular injury. The CYP3A4 expression of GS-3A4-HepG2 cell line was controlled by the EF-alpha promoter. The expression of P450 3A4 is constitutive and not inducible. Inducibility of primary hepatocytes is an important factor for drug evaluation. However, the importance of CYP inducibility in a BAL system is still unclear.

GS-3A4-HepG2 also expressed glutamine synthetase. The GS-3A4-HepG2 cells were cultivated under an ammonia concentration of 0.95 mM, and the ammonia-metabolizing activity was evaluated during the log phase. GS-3A4-HepG2 could metabolize ammonia during cultivation. The ammonia-metabolizing activity of GS-3A4-HepG2 was $(0.051 \pm 0.085) \times 10^{-13}$ mol/cell/h. In previous studies, the GS-HepG2 cell lines (200 and 300 μM MSX-tolerant cell lines), which could support pigs with liver failure, showed ammonia-metabolizing activity at $(0.14 \pm 0.017) \times 10^{-13}$ and $(0.23 \pm 0.05) \times 10^{-13}$ mol/cell/h, respectively (*7*, *10*, *11*).

In previous work, these GS-HepG2 cell lines were constructed by *gs* gene amplification under different MSX, glutamine inhibitor, concentrations (*7*, *30*). The constructed pBudCE-GS-CYP3A4 vector contained the *gs* and *CYP3A4* gene within one vector. Drug metabolism of the GS-3A4-HepG2 and GS-3A4-CHO could be expected to increase with the amplification of the exogenous *gs* gene. To increase drug-metabolizing activity by gene amplification, we selected MSX-tolerant cell lines under different MSX concentrations. After a more than 1 month selection period, we could obtain 100, 200, 300, and 500 μM MSX-tolerant GS-3A4-HepG2 cells. We evaluated the testosterone 6β hydroxylation activity and glutamine synthetase activity of these MSX-tolerant GS-3A4-HepG2 cells. However, no enhancement of either type of activity was observed. In the case of GS-3A4-CHO, we could obtain 25, 100, 250, 500, and 1,000 μM MSX-tolerant GS-3A4-CHO cells. However, the testosterone 6β hydroxylation activity among these MSX-tolerant cells was not enhanced. The *gs* gene seems not to have been amplified in these constructed cell lines. These cells might acquire MSX resistance through another mechanism (*31*).

Clearance-Based BAL Evaluation. The constructed GS-3A4-HepG2 was suitable for a bioartificial liver support system because of its high level of drug-metabolizing activity mediated by P450 3A4. In a previous experiment, we demonstrated how a bioartificial liver using our constructed GS-HepG2 cell line supported a pig with liver failure. Our constructed BAL system could prolong the survival time of the pig with liver failure. It is very difficult, however, to directly compare these results with results from other researchers' BAL support experiments because there is no quantitative parameter for BAL evaluation. Clearance is the efficiency of the organ in removing a substance from blood, and it is the most important concept for evaluation in pharmacokinetic studies (*27*). Some researchers applied pharmacokinetic models for the evaluation of BAL drug metabolism

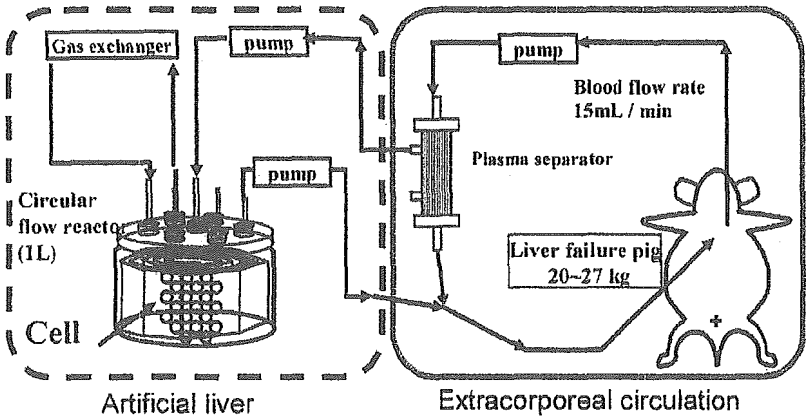

Figure 2. Bioartificial liver support system using circulatory flow bioreactor.

(*32*, *33*). Following such work, we focused on and evaluated the drug-metabolizing ability of the GS-3A4-HepG2 bioartificial liver system using a pharmacokinetic model.

Hoener predicted human hepatic clearance based on in vitro experimental data on drug metabolism (*34*). We applied Hoener's method to evaluate the bioartificial liver support system. In previous work, we used a circular flow bioreactor with glass fiber cloth as the BAL bioreactor (Figure 2) (*8*, *9*). This reactor was combined with a plasma separator and connected to the jugular vein for blood access. We assumed a well-mixed condition for the circular flow bioreactor with the plasma separator in calculations of BAL clearance. BAL intrinsic clearance, CL_{INT}, is described as eq 1. K_m is the Michaelis–Menten parameter of whole cell catalysis. R_{MAX} is the maximum drug-metabolizing activity of the BAL system and was calculated with the maximum cellular activity V_m, cellular protein content k, cell concentration X, and reactor volume V of the BAL (eq 3). C is the drug concentration of the blood; the drug is the substrate for P450 enzyme. Normally, C is constant in human blood and it could be assumed at steady-state condition. α is the fraction of drug unbound in blood. The part of drug strongly combines with plasma protein. Consequently, αC is an effective substrate concentration for enzyme reaction. Under steady-state condition, actual BAL clearance, CL_{BAL}, is a function of blood flow rate Q and BAL intrinsic clearance, CL_{INT} (eq 2) based on Hoener's equation (*34*). CL_{BAL} corresponds to the overall performance of the BAL system, and it includes cellular function V_m, cell concentration X, reactor volume V, and the rate of blood circulation from the patient. CL_{INT} corresponds to the maximum performance of the BAL system and does not include the blood flow rate. A higher clearance value means higher performance from the BAL system.

$$CL_{INT} = \frac{R_{MAX}}{K_m + \alpha C} \quad (1)$$

$$CL_{BAL} = \frac{Q\alpha CL_{INT}}{Q + \alpha CL_{INT}} \quad (2)$$

$$R_{MAX} = V_m kXV \quad (3)$$

The Michaelis–Menten parameter and maximum cellular activity of whole cell catalysis of GS-3A4-HepG2 are necessary for an evaluation of BAL clearance. Under different testosterone concentrations, the testosterone 6β hydroxylation activity of whole cell catalysis of GS-3A4-HepG2 was investigated using monolayer culture. Figure

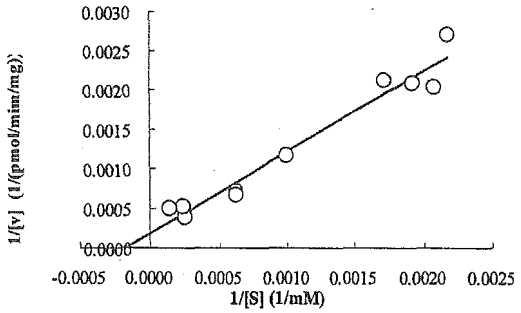

Figure 3. Lineweaver–Burk plots for GS-3A4-HepG2 testosterone 6β hydroxylation.

3 shows a Lineweaver–Burk plot of testosterone 6β hydroxylation of whole cell catalysis. From this figure, K_m and V_m were determined as having 55 ± 16 μM and 1100 ± 170 pmol/min/mg, respectively, of total cellular protein (Table 2); a 95% confidential level of kinetic parameters was obtained from linear regression analysis. Sy et al. evaluated human hepatic CYP3 enzyme kinetics using microsomal fraction (*35*). The human microsomal K_m and V_m values in testosterone 6β hydroxylation were 206 ± 48 μM and 611 ± 684 pmol/min/mg, respectively, of microsomal protein. Microsomal and whole cell results were indeed difficult to compare directly, but one finding from their comparison is that GS-3A4-HepG2 might have lower K_m and higher V_m values for testosterone 6β hydroxylation than might those of human hepatocytes. It was not considered about the limitation of membrane transport in case of measurement of microsomal kinetic parameters. Consequently, it was estimated that the K_m value of microsomal P450 activity was underestimated and the V_m value was overestimated comparing with whole cell data.

Testosterone is the principal and most potent angrogen. As a pharmaceutical, testosterone is not very important medically, but it is a typical substrate for evaluation of P450 3A4 activity. We calculated BAL clearance for testosterone metabolism using K_m and V_m values of GS-3A4-HepG2. BAL evaluation parameters for clearance calculation were obtained from our previous BAL support experiments (*8*–*11*). In our previous BAL system using the GS-HepG2 cell line, we used a working volume (V) of 1 L for the bioreactor and inoculated cells at a concentration (X) of 4 × 10^6 cell/mL. The blood flow rate (Q) was 15 mL/min for circulation from a pig with liver failure. The average protein content for GS-3A4-HepG2 was 0.48 ng of total cellular protein per cell. This value is lower than that of the primary hepatocyte

Table 2. Kinetic Parameters of Testosterone 6β Hydroxylation

	K_m [μM]	V_m [pmol/min/mg of total cellular protein]
GS-3A4-HepG2	55	1100
human primary hepatocyte[a]	206	611

[a] Estimated from microsomal parameters.

Table 3. Estimated Clearance of GS-3A4-HepG2 Cells

	testosterone 6β hydroxylation clearance	
	BAL intrinsic clearance (mL/min)	BAL clearance (mL/min)
GS-3A4-HepG2	38.4	0.66
human primary hepatocyte[a]	1.8	0.03

[a] Estimated from microsomal parameters.

(1.2 ng of total cellular protein per cell) (*36*). We assumed that the BAL was applied to treat high testosterone serum. Osredkar et al. reported a serum testosterone concentration of 2.6 nmol/L in patients with hirsutism, in which the fraction of unbound testosterone (α) was 0.018 (*36*). We calculated BAL clearance in cases using GS-3A4-HepG2 and human hepatocytes using the above parameters X, Q, and V. In the case of human hepatocytes, the K_m and V_m parameters of liver microsomes were used for this calculation because parameters of whole cell catalysis were unknown. The human hepatocyte parameter was calculated as (microsomal parameter (V_m or K_m) × (primary hepatocyte protein content (1.2 ng/cell) (*36*)) × (0.125 microsomal protein/total cellular protein (*24*)). Estimates of the clearance are listed in Table 3. The BAL system with GS-3A4-HepG2 was shown to have only 2% of intrinsic clearance. Furthermore, GS-3A4-HepG2 performance (BAL clearance) was 2.5 times that of human hepatocytes, whereas the V_m value of GS-3A4-HepG2 was about 20 times.

Effect of BAL Operational Parameters on BAL Clearance. To increase BAL performance, we simulated the effect of BAL operational parameters on BAL clearance using eq 3. In a previous study, we performed BAL support experiments at a concentration of 4×10^6 cell/mL. However, the circulatory flow bioreactor could attain a maximum cell concentration of 5×10^7 cell/mL (*38*). Figure 4 shows the effect of BAL operational parameters, cell concentration, blood flow rate, cellular activity V_m and K_m on BAL clearance. As shown in the figure, an increase in cell concentration resulted likewise in an increase in BAL clearance. In the case of the GS-3A4-HepG2 cells at a concentration of 5×10^7 cell/mL, which is the maximum cell concentration of the circulatory flow bioreactor, BAL clearance increased to 5.48 mL/min. Obviously, such a high R_{MAX} value can also be attained with higher V_m parameters of GS-3A4-HepG2. However, this constructed cell line has already attained a high activity level similar to that of human primary hepatocytes. Increasing the V_m parameters of GS-3A4-HepG2 by further recombination or gene amplification might be more difficult than increasing cell concentration. However, we assumed the monolayer parameters into high cell density cultivation. In high cell density or 3D culture, the enhancement of liver function could be expected. These enhancement is likely to increase the V_m parameter of cell line or primary hepatocyte, not K_m. Development of a 3D cultivation method could be quite effective for increasing V_m parameter. On the other hand, the blood flow rate was not an effective factor for increasing BAL clearance. Ueda et al. proposed a whole blood perfusion-type bioreactor to increase the blood flow rate into a BAL system (*39*). Their system could attain a blood flow rate of 200 mL/min. Even at this increased blood flow rate of 200 mL/min, BAL clearance increased to only about 0.69 mL/min. The blood flow rate in the human liver is about 1,500 mL/min. However, BAL clearance increased to only 0.69 mL/min at the blood flow rate of 1,500 mL/min.

It is still unknown what kind of liver functions should be supported by a BAL system. In this study, we focused on drug metabolism and constructed a drug-metabolizing BAL system because drug-induced hepatocellular injury is one of the most serious forms of hepatopathy and the number of patients with it is increasing (*28*, *29*). We constructed a high-metabolic P450 3A4 HepG2 cell line, and its capability is the same as or higher than that of human primary hepatocytes. In a clearance-based evalu-

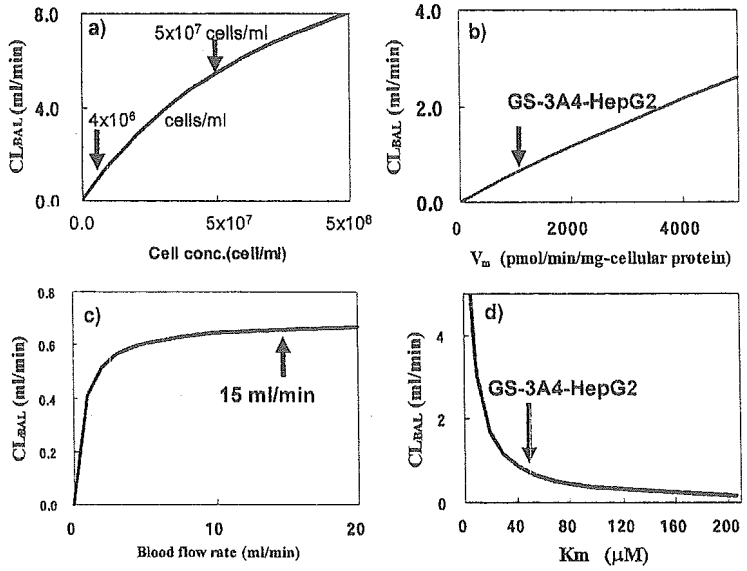

Figure 4. Effect of BAL operation parameters on the BAL clearance: (a) cell concentration, (b) V_m (cellular activity), (c) blood flow rate, (d) K_m. The arrow shows the calculation results based on previous BAL experimental parameters and kinetic parameters of GS-3A4-HepG2.

ation, the BAL system functions were limited by whole cell catalytic activity in the case of testosterone metabolism. As expected, this conclusion changes according to the blood concentration, C, and the fraction of drug unbound in blood, α. For example, lidocaine is a commonly used local anaesthetic and a typical flow-limiting drug for liver metabolism. Lidocaine is mainly metabolized by P450 1A2; for lidocaine, C is 4 μM and α is 0.3. Lidocaine metabolism in the BAL system was strongly affected by the blood flow rate. Accordingly, the effectiveness of the BAL system varied according to the characteristics of the objective drugs in the blood. From another point of view, the definition of clearance should be considered. That is, BAL clearance evaluation should be based on a drug's "disappearance" from blood by the P450 enzyme. Actually, metabolized drugs do not disappear but are converted into other compounds. Normally, such metabolized compounds are metabolized by a phase II reaction, conjugated, and excreted by the kidney. Combinations of P450 and phase II conjugation and excretion reactions will likely be necessary for drug-metabolizing artificial liver support systems. Primary hepatocytes could maintain high Phase II activity. In the case of cell line, the high Phase II activity should be required for BAL construction. Therefore, construction and co-culture of other cell lines that specialize in a phase II reaction or excretion systems by kidney cells requires further study.

Notation

k	protein content per cell (mg protein/cell)
C	drug concentration in blood (μmol/L)
CL_{BAL}	BAL clearance (mL/min)
CL_{INT}	BAL intrinsic clearance (mL/min)
K_m	Michaelis–Menten parameter of whole cell catalysis (μmol/L)
R_{MAX}	drug-metabolizing activity of BAL system (nmol/min)
Q	blood flow rate between bioartificial liver support system and liver failure mammals (mL/min)
V	total volume of bioartificial liver support system (mL)
V_m	maximum activity of whole cell catalysis (nmol/min/mg-total cellular protein)
X	cell concentration in the bioartificial liver support system (cells/mL)
α	fraction of drug unbound in blood

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体外血液浄化法の現状と人工細胞による非細胞系バイオ人工肝への期待

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The variety of extracorporeal blood purification devices and the design of non-cellullar bioartificial liver.

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One well-established treatment for hepatic and renal dysfunction is hemodialysis and plasma pherasis with ultrafiltration membrane. The treatment has been improving the therapeutic outcome, but the method is basically dependent upon simple diffusion and thus, lacks ability of active transportation and selective removal of toxic substances. To add biological activities to present hemodialysis technique, various challenges have been performed to develop bioartificial organ that is consisted of artificial materials and cells, especially hepatocytes. Indeed the bioartificial livers show certain therapeutic efficacy yet, the strategy has several intrinsic disadvantages for clinical use, such as complicated handling, inappropriateness for emergency use, risks of cell-mediated infection, etc. Thus, we have attempted to construct artificial cells by extracting cellular functional molecules on lipid layer instead of use of cells themselves. Experimentally, artificial lipid bilayer or liposome have been used to mimic cellular functions. We construct proteoliposomes that contain multidrug resistance 1 (MDR1) protein, one of ABC transporter proteins, and estimated their biological activities. More than 80% of the transporter proteins are shown to be integrated in the lipid layer inside-out by ATP hydrolysis assay and to incorporate radiolabeled substrate into the liposome. We will introduce our concept and present progress.

【キーワード】
ハイブリッド人工肝，人工細胞，プロテオリポソーム，MDR-1

はじめに

肝・腎機能がそこなわれた患者に施される血液浄化法は，限外濾過膜を使った血液透析が主流である。人工腎臓としての透析技術の研究は1900

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年代初頭から行われており，現在にいたるまで様々に形を変えてきた。しかし，生化学反応が複雑に組み合わさった臓器の補助としては，物理的作用に頼る血液透析では性能が十分とはいえず，特に様々な物質の代謝を行う肝臓の機能代替はまだ達成できていない。これを克服するためにリガンド固定カラムなどの人工材料による毒性物質の選択的除去が図られて来たが，それでも静電相互作用による吸着などの物理的作用に頼るところが大きく，選択的・能動的な物質除去能の付与は不十分である。そこで，生きた細胞をそのまま体外血液浄化法に利用し，選択的・能動的物質除去能を高めたハイブリッド人工臓器の開発が取り組まれている。これにより代謝機能は向上するものの，細胞利用によるウィルス感染のリスクや莫大なコストなどの問題があり，いまだに臨床例もわずかで，今後の汎用化は困難と考えられる。そこで，我々は細胞の代わりに，特定の細胞機能を持つ人工細胞（リポソーム）を用いた血液浄化システムの開発を始めている。リポソームは化粧品に配合されるなど，すでに実用化がなされており，今後の開発の発展により高度な品質管理や量産化もねらえると考えている。本稿では，今までの血液浄化法の歴史を振り返りながら，筆者らが考える非細胞系バイオ血液浄化システムの将来像に触れたい。

1．体外循環による非生物学的血液浄化法の現状

現在最も一般的におこなわれている限外濾過膜を使用した血液浄化法は，物質の拡散浸透の現象を利用した物理的な毒物除去法である。人工腎臓としての血液透析の技術は非常に古くから研究されており，1913年，Abelらがコロジオン膜を用いて血液の体外循環による透析の可能性を動物実験で実証したとされる[1]。1938年にはThalhimerによってセロファン膜を透析膜に利用した血液透析が実験されている[2]。人工腎臓としての体外血液浄化法が飛躍的に発展・普及したのは1950年の朝鮮戦争のころである。激しい負傷によって発症する挫滅症候群による腎不全を起こした兵士に人工腎臓が利用されたのである。これにより患者の死亡率を半減させたという[3]。その後数多くの改良[4,5,6]がなされ，現在主流となっている中空糸膜（ホローファイバー）による透析は，1967年，LippsとStewartによって開発された[7]。中空糸膜を多数束ねた形状の血液透析器は，血液の接触面積が高く，小型で，効率よく毒物除去ができるよう工夫されている。しかし原理的には膜の分画分子量に依存したものであるため除去物質の選択性に乏しく，目的の毒物の除去が不十分であるなどの問題が残る。そこで，透析膜だけに頼らない血液浄化法として，アルブミン透析や血漿吸着法が考案された。

アルブミン透析は，ビリルビンなどのアルブミン結合性の毒性物質を除去するもので，透析液にアルブミンを添加して，血液中の毒物を外液側に移動させている。これを実用化したのは，Teraklin社（独）のMolecular Adsorbent Recycling System（MARS）であり，すでに臨床治験も多数行われている[8]。さらにMARSの特長としては，除去された毒物をイオン交換樹脂や活性炭によってアルブミンから除去し，アルブミン液を再利用するシステムを実現している。

また血液中から血漿を分離除去し，正常な血漿と交換して毒物を除去する血漿交換法は現在広くおこなわれているが，血漿の必要性，血漿由来の感染症のおそれなどの問題がある。この問題を解決するために，分離した血漿から吸着担体を利用して毒物だけを除去し，浄化された血漿を体に戻す血漿吸着法がある。吸着担体としては高分子材料などを基盤とし，目的の除去物質に応じたリガンドを固定化して静電作用や疎水性相互作用などにより選択性を付与している。たとえば，4級塩化アンモニウムをリガンドとしてビリルビンや胆汁酸の吸着を狙ったものや（例：プラソーバ・旭メディカル製），ヘキサデシル基をリガンドとしてβ2ミクログロブリンの除去を行うカラム（例：リクセル・鐘淵化学製）などがある。他にもこのようなアフィニティを利用したカラムとして，

重症筋無力症の原因抗体である抗アセチルコリンレセプター自己抗体を除去するための合成ペプチドリガンドカラム（例：メディソーバMG-50・クラレ製）もある。またリガンドを用いず，セラミックなどの多孔質吸着材料で除去物質の分子サイズに応じて空隙を制御し，除去物質の選択性を付与した材料も研究されている[9]。

2．細胞を利用した体外型バイオ血液浄化システム

前述のように，血液浄化における透析膜，分離担体について，これまで様々な材料が研究され，目的に応じたデザインの血液浄化装置が開発されている。しかしながら物理的除去の域を越えられず，除去物質の選択性，能動性，すなわち濃度勾配等に依存しない物質移動をおこなえるという点では生体に遠く及ばないものである。この問題を解決すべく，細胞の機能をそのまま血液浄化に用いた，バイオ人工臓器の開発が進められている[10]。

細胞ないし組織を利用した人工的な肝機能補助装置の研究は，日本では1950年代から既に始まっていた。1956年から1958年にかけて，生きたイヌの循環系に肝性昏睡患者の血流をつなげ血液浄化に用いたという報告がなされ，一部の患者では昏睡からの覚醒という効果が見られたとされる[11, 12]。さらに，1960年代には摘出した異種動物肝に患者の血液を通す方法も試みられた[13, 14]。しかし，動物の肝臓をそのまま用いると，即時的な強い免疫反応により血管が詰まる問題が起こる[15]。そこで，人工材料で免疫隔離膜を形成し，抽出した肝細胞を組み込んで利用する方法がとられはじめた[16-20]。これらはハイブリッド人工肝またはバイオ人工肝とよばれるが，後者の頭文字をとったBAL（Bioartificial liver）という名称が欧米では一般化しているので，本稿でもバイオ人工肝の語を用いる。

肝細胞の形質・機能維持には，培養状態が大きく影響を与える。培養方法は，初期の頃は浮遊培養系が用いられたが，細胞の活性維持向上のために，細胞を基材に固定して用いられるようになった。例として単層培養[16]，包埋培養[17]，マイクロキャリアー法[18]，ホローファイバー培養[19]などがある。最近では，オルガノイド肝細胞培養モジュールが開発され，それにより2ヶ月以上の細胞の機能保持を達成している[20]。

アメリカでは，ベンチャー企業HEPATIX社によって，初めて不死化ヒト肝細胞を使用した体外肝臓支援装置ELAD（商標 Extracoporeal Liver Assist Device）が開発され[21]，その後VitaGen社がこれを改良し，カートリッジ製造の工場を設立した。2003年には，同社の資産と業務の権利を得たVital Therapies Inc（VTI）がELADの市場参入を図っている[22]。また我々は，以前に阻血性肝不全ブタを用いたバイオ人工肝（回流式バイオリアクター・キグナス）を作製し，ブタの血液から血漿を分離し，カラム内に播種した機能性細胞によって血漿内のアンモニアを代謝させ，有意な生存延長効果が得られることを確認した[23]。バイオ腎臓の研究は，バイオ人工肝ほど数は多くないが行われており，たとえば尿細管上皮細胞を播種した中空糸によって，効率的な持続ろ過ができる可能性が得られている[24]。

このようにバイオ人工臓器の開発は様々な形で行われているが，細胞の利用にあたっては，操作の煩雑性，ウィルス感染の危険性，要事対応性の欠如，品質管理の困難さやコスト高になるため量産ベースに乗せにくい，などの難点が残る。実際，数多くの研究が行われているにも関わらず，臨床例はわずかである。そこで，我々は，生きた細胞の代わりに細胞の機能を抽出し，人工細胞（リポソーム）を構築するという方法でこれらの問題を解決しようと試みている。

3．リポソームの歴史と種類

生体膜は脂質，タンパク質および糖類から成り立っていることはすでに教科書的な知識であるが，そもそも生体膜の基本骨格が脂質二重層であることを発見したのは，GorterとGrendel（1925年）である[25]。これは赤血球から抽出した脂質を単分子膜にした際，表面積が赤血球の2倍あることか

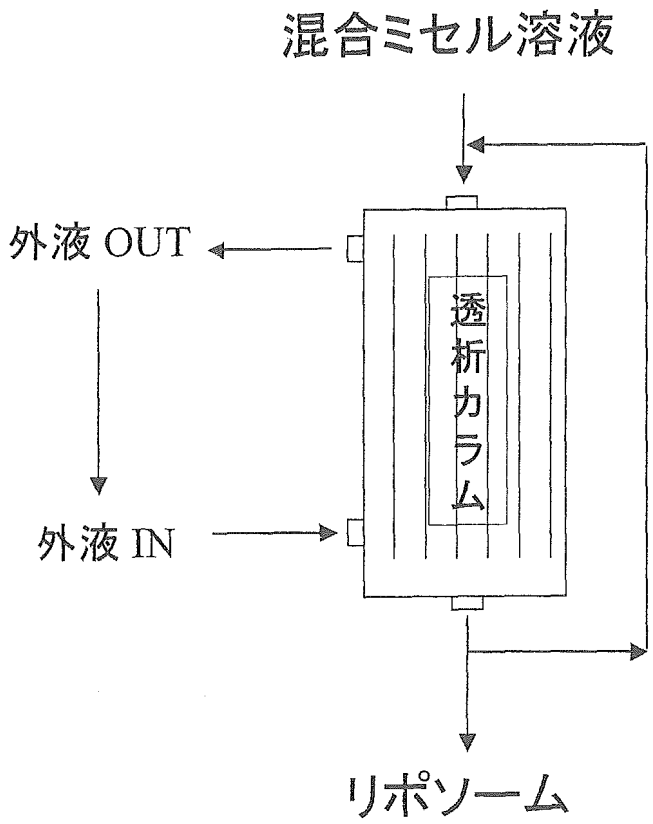

図1　血液透析カラムを利用したリポソーム作製法の模式図

ら得られた結果であった。また1964年，イギリスのBanghamとHorneは脂質分子を水に分散させると，まるで細胞膜のような膜小胞が形成されることを電子顕微鏡で確認した[26]。

リポソームは，様々な手法で調製でき，その方法によって多重層リポソーム（MLV），一枚膜リポソーム（SUV），大きな一枚膜リポソーム（LUV）などが調製できる。この中でもMLVが最も簡単に調製でき，脂質を溶解した有機溶媒を除去してできた脂質薄膜に水を加えて攪拌することで得られる。このMLVを超音波照射したり，押し出し機（エクストルーダ）でMLVに圧をかけるなどしてSUVができる。ほかにもコール酸などの界面活性剤と脂質との混合ミセルから透析やゲルろ過により界面活性剤を除去する方法や，エタノールに溶解させた脂質を水溶液中に注入する方法でもSUVは得られる。LUVの調製法は，おもにカルシウム融合法，凍結融解法などがある[27]。また，脂質の種類や比率をかえることで，様々な特性をもつリポソームができる。このようにして，目的に応じたリポソームを調製することができる。我々は，SUVをつくる新規な方法として，血液透析に使用される中空糸膜を用いて，より迅速・連続的なリポソームの大量調製法を開発した。これにより，直径100 nm前後のリポソームを容易に調製して実験に用いている（図1）（特願2004-33439）。

4．リポソームの応用技術

前述のように，リポソームは，生体膜に構造が似ていることから，生体膜モデルとして様々な研究に利用されている。またその閉鎖性から，薬物

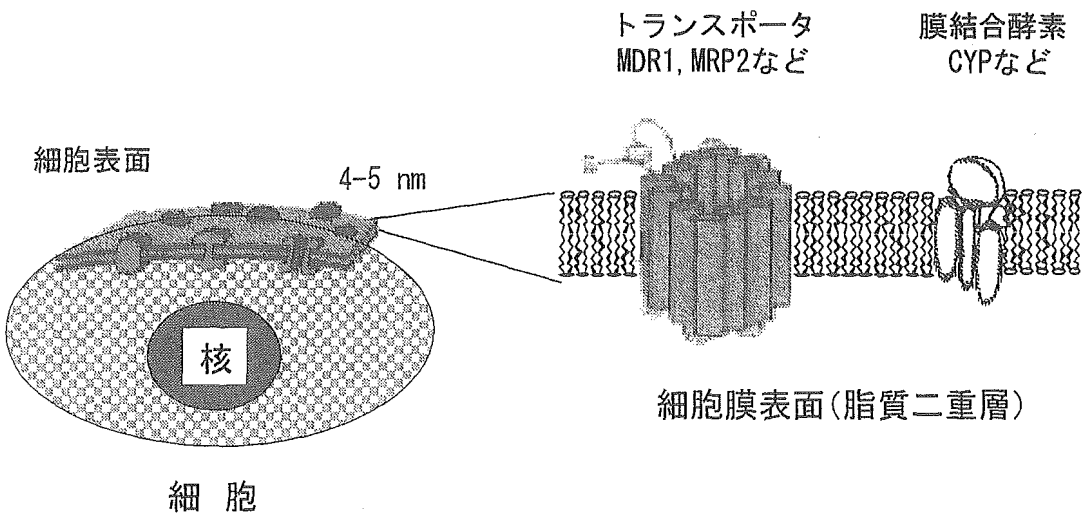

図2　細胞表面の機能性タンパクの組込み例。

A：トランスポータータンパク（MDR 1，MRP 2 など）。
B：CYP 及び電子伝達複合体，ビリルビン抱合酵素など

のキャリアなどにも多用されている。

医薬の分野では，おもに DDS すなわち Drug Delivery System のカプセルとして利用される。リポソームをカプセルに使用する利点は，低毒性，代謝により排出できる，大きさや膜透過性の制御が比較的容易，表面修飾により到達効率を高めることができる，などである。これらは癌の化学療法，感染症治療，湿布剤などに用いられる。治療薬のほかにも，リポソームに抗原を組み込んだものは診断薬として利用されてきており，RIA 法や ELISA 法に比較して操作が簡単であることが長所である。医薬品のみならず，リポソームと肌との親和性の高さから化粧品にも利用されている。すなわちリポソーム中に有効成分を封入し，日焼け止めや化粧水，デオドラント製品等として販売されている。また，感熱応答性を付与して所定温度で内包物を放出させるように制御したものもあり，今後の応用が期待されている[28]。

さらに，リポソームによる遺伝子導入技術も実用化されている。遺伝子導入法としてマイクロインジェクションやリン酸カルシウム法があるが，これらは効率が悪く，*in vivo* で応用できない。しかし，リポソーム法は導入効率に優れ，*in vivo* での系に利用できる[29]。またカチオニック（陽性荷電）リポソームを使用することで導入効率が高まることも分かっている[30]。最近ではバイオサーファクタントとよばれる微生物由来の界面活性物，例えば mannosylerythritol lipid という糖型脂質からなるリポソームにより飛躍的に導入効率が上がったという報告がある[31]。

血液浄化にリポソームを利用する研究も行われている。リポソームとビタミン C，ビタミン E を組み合わせたもので，脂質やタンパクの酸化ストレスの抑制や，ビリルビンの除去効率向上などの効果が出ている[32]。

5．リポソームによる人工細胞の構築

前述のように，生体膜とリポソームの形状が非常によく似ていることから，リポソームは細胞膜モデルとして広く利用されている。最近の興味深い研究では，微小管タンパク質を導入したリポソームによる，細胞骨格系の機能解析の研究があ

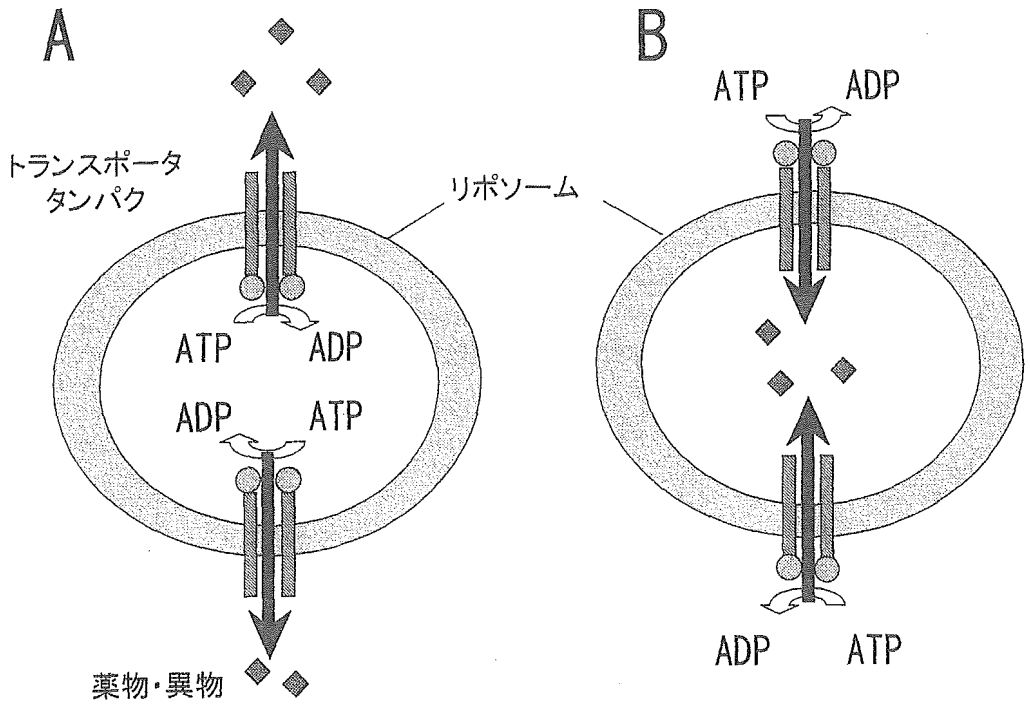

図3　トランスポータータンパクの脂質膜上での配向のモデル図。

A：細胞表面における配向（外向き・生理的配向）。トランスポーターが毒物を膜外へ排出する様子。
B：プロテオリポソーム上での配向（内向き）。トランスポーターが毒物を膜内へ取り込む様子。

る[33]。

細胞機能研究のほかに，医療に利用される人工細胞も開発されている。一例として，ヘモグロビンを内包し酸素運搬機能を持たせたリポソームがあるが，これは人工赤血球と言う点で人工細胞の一種といえる。我々は人工血液浄化システムの一部としての人工細胞の開発を行っている。我々が毒物代謝機能をもつ人工細胞を造るために注目したのが，細胞内の毒物を細胞外へ輸送するトランスポータタンパクである。

現在モデルタンパクとして用いているトランスポータータンパク（図2）は，細胞内の脂溶性毒物を外へ排除するはたらきを持つMDR（Multi Drug Resistance）-1とよばれる膜タンパクである。これは，細胞膜内にATP結合ドメインを持ち，ATPの加水分解エネルギーによって機能を制御しているABCタンパク質のひとつであり[34]，生体内では小腸，脳毛細血管，腎尿細管等に発現し，細胞内に侵入した異物を能動輸送により体外へ排出させている。

このMDR-1は，癌細胞の多剤耐性にも深く関与している。MDR-1が過剰に発現したガン細胞では，抗癌剤が細胞外へ排除されてしまうのである。これは本来MDR-1を発現している組織の腫瘍によく見られる現象であり，抗がん剤が効かなくなる原因のひとつである。

このように，MDR-1タンパクは，生理的には細胞内の低分子異物を血中に排除する機能を有する。しかしながら，我々が考えるように血液浄化法に利用する為には，逆に血液中の毒物を膜内に取り込む必要がある（図3）。MDR-1などのATP依存性トランスポーターはATP水解活性

表1　代謝系人工臓器開発のあゆみ

非バイオ人工臓器		
1913	Abel	コロジオン膜を用いて血液の体外循環による透析を実験[1]
1938	Thalhimer	セロファン製透析膜を用い透析効率向上[2]
1943	Kolff	回転円筒型透析装置を開発，臨床へ[3]
1960	Kill	Kill型人工腎臓の完成[4]
1967	Lipps	中空糸型の人工腎臓の開発[6]
1976	Opolon	PAN膜による透析の実施[5]
バイオ人工臓器		
1956	杉江・堀	イヌ肝灌流システムとイオン交換樹脂の併用[11]
1958	三上・水戸	イヌ肝スライスを用いた装置の試作[12]
1958	Otto	ブタ摘出肝を用いた体外灌流の開発[13]
1994	Rozga	ブタ肝細胞を付着させたマイクロキャリアと中空糸膜，活性炭による肝疾患患者の治療[18]
1994	Sussman	ヒト肝細胞を固定した中空糸膜を用いた治療の開発[19]

表2　アフィニティ透析で除去できると考えられる標的物質とその
トランスポーターやリガンド

毒性物質	トランスポータ・リガンド
有機アニオン薬剤	MDR-1
ビリルビン	ABCA8
低分子外来異物	CYP3A4
グルクロン酸抱合体形成	UDP-glucronosyltransferase
β2-microgloblin	Megalin

部位を細胞内に有する。そこで，MDR-1を組み込んだプロテオリポソームによるATPase活性測定によりリポソーム膜表面でのMDR-1の配向性について詳細に調べた。そしてMDR-1がほぼ目的の向き，すなわちリポソームの内腔に向かって基質を取り込むように配向していることを確認した。

さらに我々は非細胞系のバイオ血液浄化法の開発という観点から，作製したプロテオリポソームがMDR-1の本来の生理機能である毒物輸送能を行うかについて調べた。MDR-1の基質である放射性ジゴキシンを加えると，脂質だけで構成したリポソームと比較して，プロテオリポソームではATP存在下での取り込みが数倍に上昇することがわかった。すなわちプロテオリポソームに存在するMDR-1がATP加水分解エネルギーを利用して，モデル毒物を膜内部に輸送・除去したことが確認できた。

おわりに

臓器機能の代替を担う人工臓器の開発の発展とともに，より生体に近い代謝を行うべく，生きた細胞と人工材料を組み合わせたハイブリッド人工臓器の開発が進められている。しかし，前述したように，緊急性に対応できるか，ウィルス感染の予防は大丈夫かなど問題は多い。一方で，細胞の必要な機能を模倣した人工細胞（プロテオリポソーム）を用いることで，これらの問題は解決され

るかもしれない。最近では，細胞やタンパク質の機能を人工的に設計し，再現しようとするバイオミメティック研究も活発に行われている。これらの技術を組み合わせて，血液浄化のみならず，様々な疾患治療に応用できる可能性が高いと考えている。

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