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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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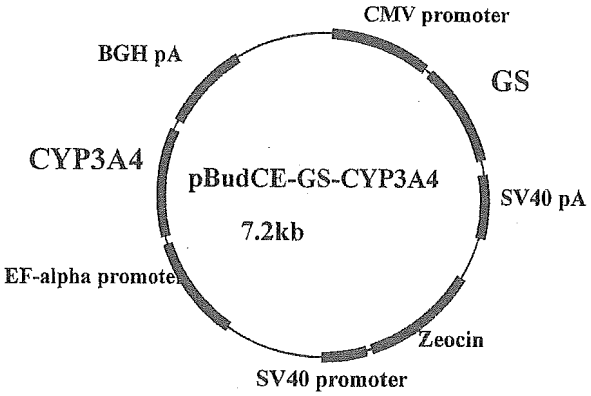

Figure1. pBUdCE-GS-CYP3A4 vector

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′-TTTTTTGCGGCCGCGTGA**TG**GCTCTCATCCCAG-ACTTGG-3′ (upstream primer containing a *Not* I site), and B, 5′-GGGGGGAGATCTATTCAGGCTCCAC**TTA**-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′-AAAAAAAAGCTTACC**ATG**GCCACCTCAGCAAGTT-CCC-3′ (upstream primer containing a *Hind* III site), and D, 5′-CCCCCCGGATCCAA**TTA**GTTTTTGTATTGGAA-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

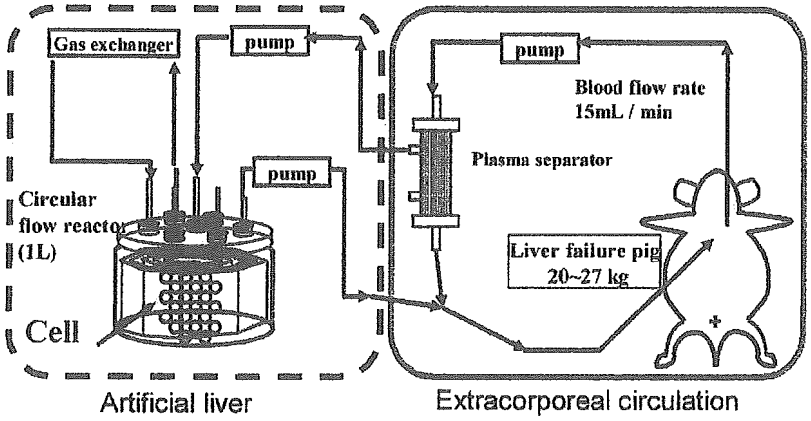

Figure 2. Bioartificial liver support system using circulatory flow bioreactor.

(*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 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.

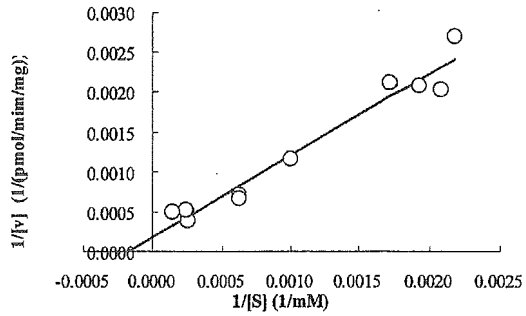

Figure 3. Lineweaver–Burk plots for GS-3A4-HepG2 testosterone 6β hydroxylation.

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-

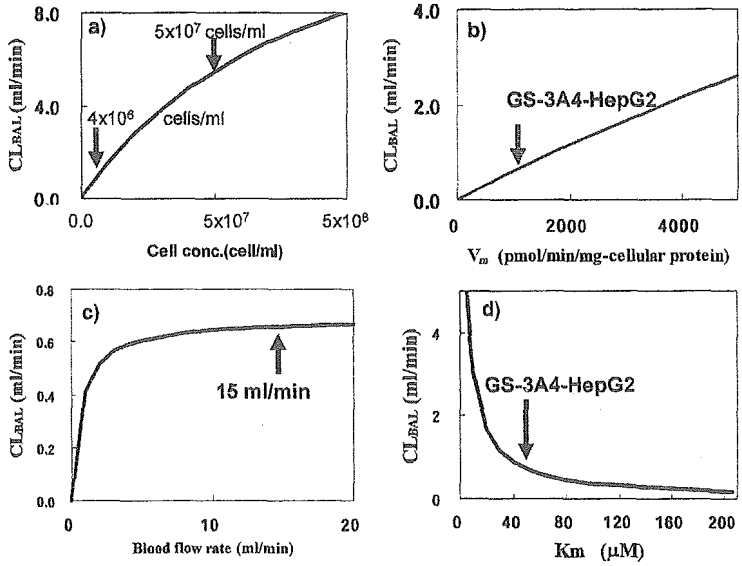

Figure 4. Effect of BAL operation parameters on the BAL clearance: (a) cell concentration, (b) V_m (cellular activity), (c) blood flow rate, (d) K_m. The arrow shows the calculation results based on previous BAL experimental parameters and kinetic parameters of GS-3A4-HepG2.

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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Development of Cell-based simulator system for drug evaluation

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Abstract

In general, biotransformation reactions in the human liver generate more polar, inactive metabolites that are readily excreted from the body. However, in some cases, metabolites with potent biological activity or toxic properties are generated. Consequently, biotransformation of xenobiotics in the human liver is important evaluation criteria for the toxicological and pharmacological studies during development of pharmaceuticals. The main reaction of biotransformation is hepatic P450 monooxygenase system. Especially, the induction and expression of P450 enzyme play a most important role for human drug metabolism. In this study, we constructed the xenobiotics-inducible cell line for simple cell-based drug evaluation and investigated the drug evaluation using micro-scale flow cell culture system.

Of the approximately 1000 currently known P450s, about 50 are functionally active in human beings. Among these P450s, the P450 3A4 is most widely expressed and shares 30% of total P450 enzyme in humans. Moreover, about half of ethical pharmaceuticals were metabolized by P450 3A4. We constructed the p3A4cp-EGFP vector which contains the *CYP3A4* cDNA and *EGFP* genes under CYP3A4 promoter. The human liver-derived HepG2 cells were transformed by this vector and the xenobiotic inducer for 3A4 was added in the medium. The green fluorescence was observed in the presence of xenobiotic inducer. We are now combining the drug-metabolizing cell line and micro-scale flow bioreactor for constructing simple drug evaluation system.

Introduction

Biotransformation of xenobiotics in the human liver is important evaluation criteria for the toxicological and pharmacological studies during development of pharmaceuticals. Drug biotransformation reactions are classified as either phase I functionalization reaction and phase II biosynthetic (conjugation) reactions. Phase I reaction introduces or expose a functional group on the parent compound. Phase I reactions generally results in the loss of pharmacological activity, although there are examples of retention or enhancement of activity. The main reaction of phase I is hepatic P450 monooxygenase system. Especially, the induction and expression of P450 enzyme play a most important role for human drug metabolism.

Of the approximately 1000 currently known P450s, about 50 are functionally active in human beings. Among these P450s, the P450 3A4 is most widely expressed and shares 30% of total P450 enzyme in humans. Moreover, about half of ethical pharmaceuticals were metabolized by P450 3A4. In this study, we constructed liver-derived HepG2 cells which were transformed by *CYP3A4* cDNA and reporter genes for drug evaluation.

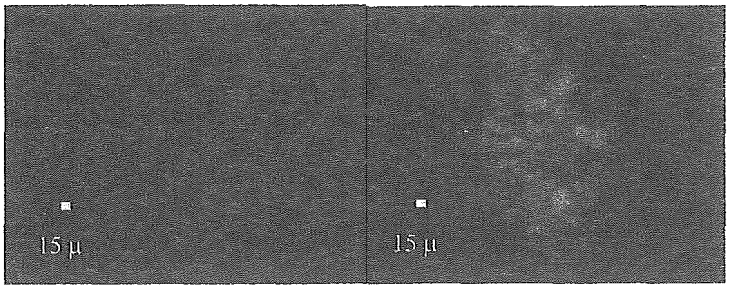
Materials and Methods

HOST CELL LINE AND CONSTRUCTION OF RECOMBINANT CELL LINES

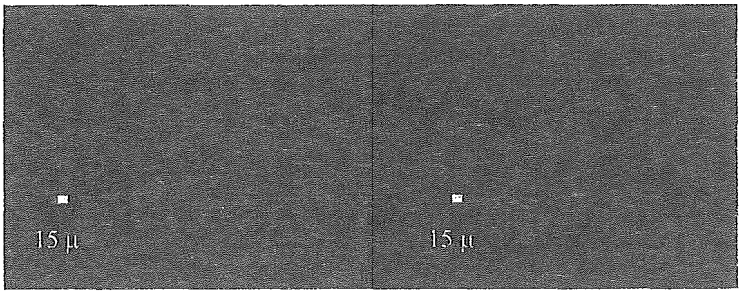
The host cell line employed in the experiments was HepG2 (RCB0459). The p3A4c-EGFP vector was constructed by insertion of the *CYP3A4* cDNA gene into the pIRES2-EGFP vector. The expression vector, p3A4cp-EGFP was constructed from the exchange of CMV promoter to CYP3A4 promoter of p3A4c-EGFP vector. The expression vector, p3A4cp-d2EGFP was also constructed from pIRES2-d2EGFP vector using same procedure. The HepG2 cells were transformed with the p3A4c-EGFP or p3A4cp-d2EGFP vector by lipofection. The recombinant cells were selected using G418 containing RDF medium.

Results and Discussion

The HepG2 cells were transformed with the p3A4c-EGFP or p3A4cp-d2EGFP vector by lipofection. The 10μM xenobiotics, clotrimazole, dexamethasone, phenytoin and rifampicin which are the inducer for CYP 3A4 were added into the medium. In case of p3A4cp-d2EGFP transformed HepG2 cell, the cells responded to the xenobiotics.



Dexamethasone



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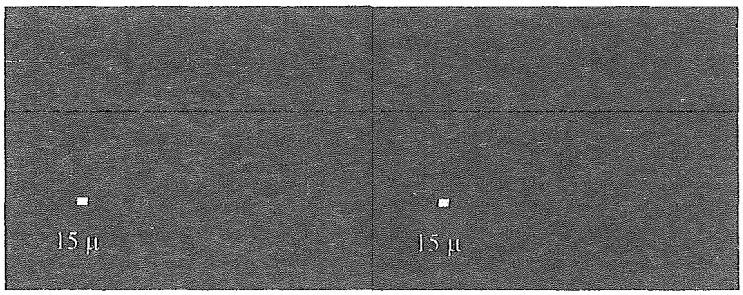
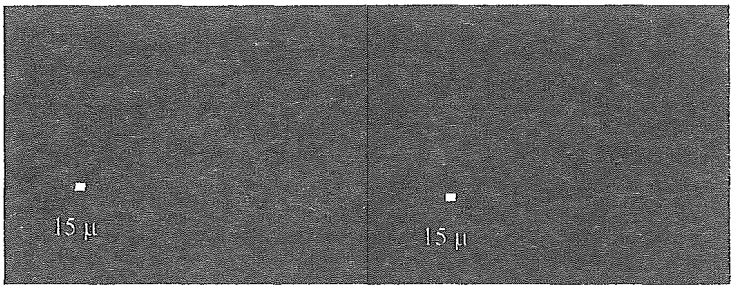


Phenytoin



Rifampicin

Fig.1. The xenobiotics induction in p3A4cp-d2EGFP transformed HepG2 cell (day 3);
Left; phase contrast microscope, Right; fluorescence microscope

The green fluorescence was observed in the presence of xenobiotic inducer. We are now combining the drug-metabolizing cell line and micro-scale flow bioreactor for constructing simple drug evaluation system.

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バイオ人工肝臓の評価は如何にあるべきか？

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1．バイオ人工肝臓とは

ハイブリッド型バイオ人工肝（Hybrid Bio-Artificial Liver Support System：BAL）とは、人体中の化学反応を担う肝臓を代替えする手段の一つとして、肝機能の中心を担う部分に生物学的素材を用いた人工臓器である。バイオ人工肝は、肝臓の機能を持つ細胞を肝補助を担う媒体として用いることから、細胞容器としてのバイオリアクターの人工肝への応用が試みられ、非常に様々な種類の人工肝リアクターが構築されている。近年では、肝臓の機能を担う細胞として、これまでよく用いらてきた初代肝細胞以外に、遺伝子組換えによって機能強化をした細胞、さらには各種幹細胞、ES細胞等から分化することにより肝機能を持たせた細胞等がソースして検討されてきている。また、リアクターについても従来の細胞培養装置から発達した体外循環型・高密度培養装置のみならず、生体内に埋め込むことを意識した生分解性素材を用いて構築されるマイクロファブリケーションを利用したリアクターも開発されてきており、上記の新たな細胞ソースと組み合わせて肝臓を再構築する手段は様々に広がってきている（図1）。

一方、この様なバイオ人工肝臓の高機能化の一方、本来サポートする対象である肝臓自身の機能が複雑かつ多岐にわたるため、これを代替えする人工肝自身に必要とされる機能がユーザーサイドから絞り込まれておらず、未だに明確ではない。そのため、選択肢は広がっているものの、バイオ人工肝臓を設計や評価するにあたってどの様な基準が用いられるべきであるかについては、十分な議論・検討がほとんどなされていない。本稿では筆者らが行ってきたバイオ人工肝に必要な評価について紹介し、問題提起としたい。まず、設計基準については、ハイブリッド型人工肝による薬物代謝に焦点を絞って、代謝系臓器再構築に必要な設計基準としてのクリアランス

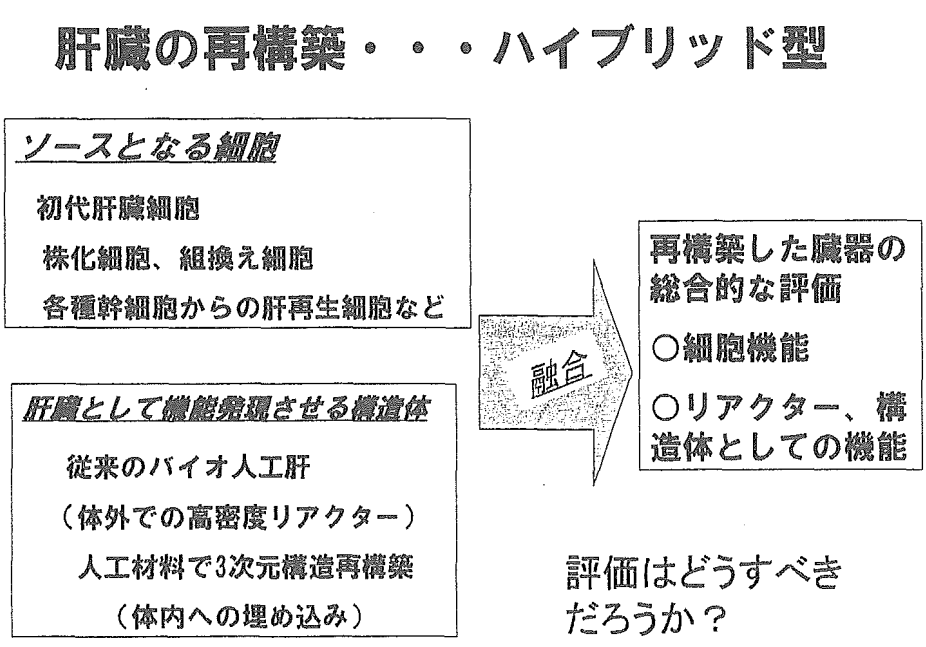

図1　バイオ人工肝構成要素と評価

表1　testosterone 6β-hydroxylation活性の比較

細胞株	Testosterone 6β-hydroxylation活性 (pmol min^{-1} $mg\text{-}protein^{-1}$)
HepG2	0.6±0.05
GS−3A4−CHO	21±1.3
GS−3A4−HepG2	490±10
GS−3A4−HepG2(80日間継代培養後)	426
Human liver[3]	1000〜1500
Human primary hepatocyte[4] (分離後24h)	253
Human primary hepatocyte[5] (分離後96h)	11

表2　推定されたtestosterone 6β-hydroxylationクリアランス

Cell	Testosterone 6β-hydroxylationクリアランス (mL/min)	
	バイオ人工肝固有クリアランス	バイオ人工肝固有クリアランス
GS-3A4-HepG2	38.4	0.66
Human primary hepatocyte	1.8	0.03

について紹介する。具体的な対象として、薬物代謝を担うヒトCYP3A4遺伝子を組み込んだCYP3A4-GS-HepG2細胞を用いたバイオ人工肝について、人工肝代謝能力に及ぼす血流量、細胞活性等の影響について考察し、人工肝に必要な機能について定量的に検証する。さらに、評価に関する試みとして、階層分析法：AHP（Analytical Hierarchy method）を用いたバイオ人工肝全体の専門家評価の基準化を紹介する。

2. クリアランスを用いた薬物代謝能評価

これまで筆者らは、アンモニア代謝能を持たせたGS-HepG2細胞（RCB1681）を構築し、これを用いて、肝不全ブタへの肝補助実験を行い、延命効果を得ている[1]。さらに、薬物代謝能を付与したHepG2細胞株の構築を試み、ヒトCYP3A4遺伝子とGS遺伝子を組み込んだ発現ベクターをHepG2に形質転換し、ヒト初代肝と同レベルの活性を長期間維持可能な細胞株GS-3A4-HepG2を構築した[2]。**表1**は、P450 3A4に特異的な代謝活性であるtestosterone 6β-hydroxylation活性を測定、比較した結果を示している。初代肝臓の活性は、生体外に分離後、急速に失われるため、正確な比較は難しいため、ここでは、文献値と比較している。

この表1に示されるように、構築したGS-3A4-HepG2株は、細胞含有蛋白質あたり、単位時間あたりの代謝活性において、初代肝細胞と同レベル以上と推定される能力を比較している。では、果たしてこの細胞を用いてバイオ人工肝を構築した場合、どの程度のレベルの機能を発揮できるのであろうか。また、その発揮した機能は初代肝と比較したりすることは可能なのであろうか。これまでは、動物実験による実証以外はあまり試みられていない。しかし、工学的な観点からは人工肝の構成要素である細胞やリアクターの性能、操作因子が判明することにより、バイオ人工肝全体の能力が推定・設計可能とされる必要がある。Iwataらは薬物代謝評価に用いているクリアランスを人工肝の性能評価に用いている[6]。そこで、我々もクリアランスに着目し、バイオ人工肝全体を臓器と見なした場合のクリアランスを定義し、これを用いた評価について検討を行った[2]。クリアランスとは単位時間あたりに臓器が完全に処理できる血液容量と定義でき、mL/minの値で評価される。値が大きければ、臓器の活性も高い。バイオ人工肝においてもクリアランスは人工肝システム全体において完全に処理できる血液容積と定義することで、試算可能である。試算にあたっては、Hoenerが提唱している細胞のV_m, K_mの値を用いた以下の(1), (2)式に基づいて計算した[7]。

$$CL_{INT}=\frac{V_m}{K_m+aC}$$　(1)バイオ人工肝固有クリアランス

Testosteroneクリアランス

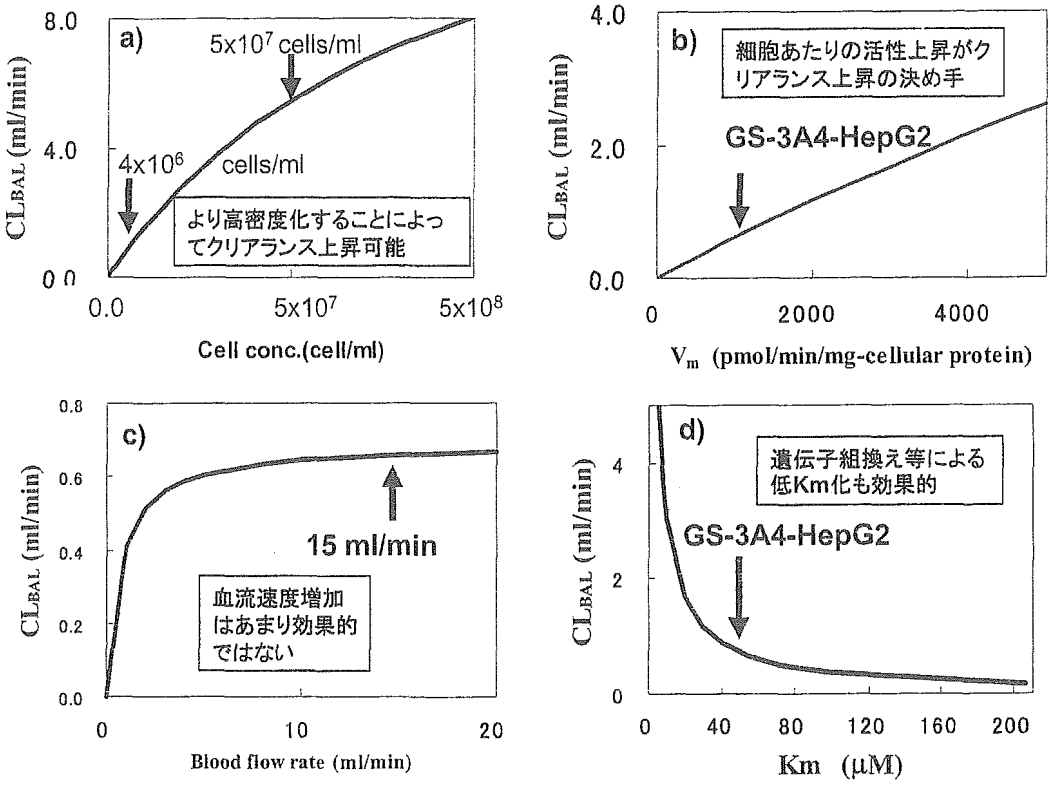

図2　各種パラメータのクリアランスに及ぼす影響

$$CL_{BAL}=\frac{Q\alpha CL_{INT}}{Q+\alpha CL_{INT}}$$

(2)バイオ人工肝クリアランス

バイオ人工肝のクリアランスを計算するにあたり、GS-HepG2細胞(RCB1681)を用いた肝補助実験において実際に利用したパラメーター(操作因子)を元にクリアランスを推定した。用いたパラメーターとして人工肝内の細胞数を4×10^9cells、血流量を15mL/min、容量1L，とした。

【Nomenclature】
CL_{INT}：人工肝固有クリアランス(mL/min)
CL_{BAL}：人工肝クリアランス(mL/min)
V_m：細胞あたりの薬物代謝活性(nmol/min/mg-protein)
K_m：Michaelis-Menten定数
C：血中薬物濃度
α：血中非結合率
Q：血流速度(mL/min)

さらに、血中テストステロン濃度C＝2.6nmol/L，血中非結合割合αを0.018とした場合の、代表的な3A4特異的代謝であるtestosterone 6β-hydroxylationクリアランスを、初代肝を用いた場合とGS-3A4-HepG2株を用いた場合を比較した(表2)。計算にあたっては、ヒト初代肝のV_m，K_mの値をミクロソーム画分の値より推定した。この表に示されるように、初代肝を用いた場合に比較してtestosterone 6β-hydroxylationに限っては、クリアランスとして約20倍の代謝能力をもっていることがわかる。

では、このクリアランス値はバイオ人工肝の操作因子であるどのパラメーターによって影響されるのであろうか。パラメーターとして操作可能な細胞濃度、血流速度、細胞あたりの活性、細胞のK_m値のクリアランスに対する影響について検討したのが図2である。

Testosteroneクリアランスの場合は、人工肝に流入する血流速度を上昇させてもクリアランスをさほど上昇さ

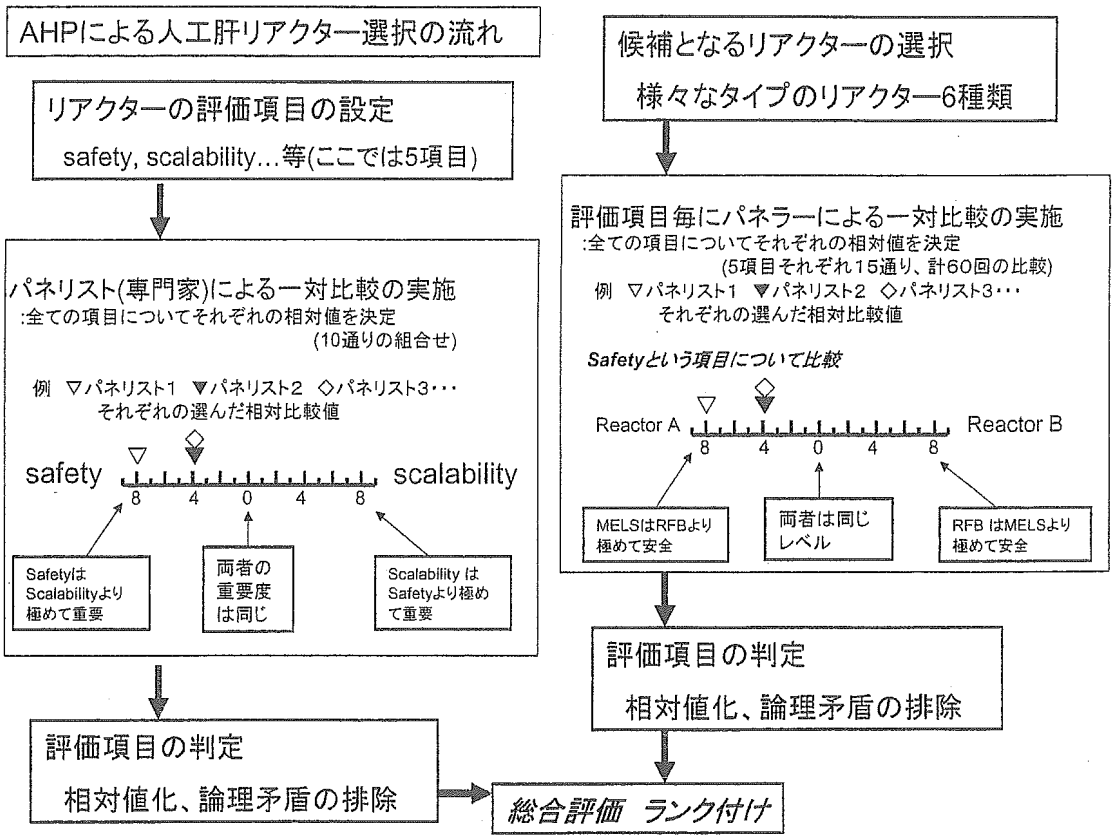

図3 AHPによるバイオリアクター判定のフローチャート

せることはできない（図2c）。すなわち、血流速度をいくら上昇させても、代謝側が律速であるために、クリアランスは上昇しない。パラメーターの根拠としたGS-HepG2細胞を用いた肝補助実験においては細胞濃度は4×10^6cells/mLとさほどリアクター内の細胞密度は高い状態ではない。ここで用いると仮定しているリアクターは5×10^7cells/mLレベル達成の実績があり、さらなる細胞密度上昇によってクリアランス上昇が図れる（図2a）。また、同様の効果は細胞あたりの代謝活性を上昇させる方法によっても達成可能（図2b）であるが、本細胞株の活性が初代肝レベルまで高まっていることから、本手法の達成は難しいと考えられる。さらに、K_mをさらに引き下げることによっても劇的なクリアランス上昇を図れる（図2d）が、こちらも手法としては難しい。血中薬物濃度C、結合率α、人工肝に用いる細胞のK_m, V_mは目的とする薬物や用いる細胞によって変化する。すなわち、対象とする薬物によって必要とされる細胞種、量、さらには操作因子としての血流速度の影響が異なり、それに応じた人工肝設計、パラメーター設定が必要とされる。また、このクリアランスの評価は血中薬物の代謝といった観点からのみ考慮されており、肝臓における第I相反応に引き続く第II相反応やその先の腎臓における排泄といった観点から考慮されたものではない。従って、血中薬物代謝という面に絞っても評価パラメーターを一元化するのは難しいのが現状である。

3．AHPを用いたバイオ人工肝総合評価の試み

上記では、バイオ人工肝の性能の中でも、薬物代謝速度（クリアランス）項目に絞った評価・設計について述べた。ところが、肝臓の機能は数百種類に上り、バイオ人工肝の性能の中には、数字を持って相互に評価するのが難しい項目もある。例えば、クリアランスと、リアクターとしての簡便性や操作性は、互いにどちらが重要か、

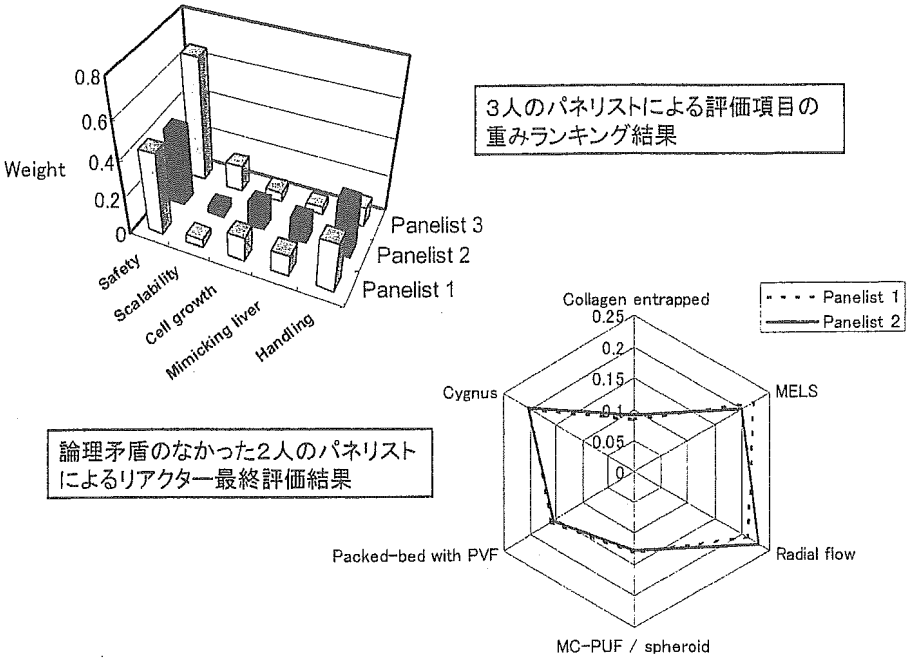

図4　AHPによる解析結果

人間が「主観的に」判断することは簡単であるが、数値を用いて「客観的に」判断するのが難しい。本稿では、オペレーションリサーチなど、経済分野での人間の意志決定の手法（補助）として応用されている階層分析法（AHP：Analytical Hierarchy method）[8]（人間の主観的な判断を数値化して比較する手法）をバイオ人工肝評価に用いた試みを紹介する[9]。

本手法の特徴は、まず問題（この場合はバイオ人工肝に適したリアクター選択）を設定し、これに対して評価項目を作成する。そして、この評価項目をパネリスト（専門家）によって「一対比較」し、それぞれをランクづける。すなわち、専門家の相対的な評価を数値化し、解析することにより、評価項目ごとの重み付けが可能となる。さらに、この評価項目毎に候補となるリアクターについて、同様の一対比較を行い、その結果と評価項目ごとの重み付け結果を総合評価することで、候補となるリ

表3　AHPによる評価項目[8]

評価項目	
Safety(安全性)	Asepsis, sterilization, immunoisolation, blood-cell separation, prevention of contamination(無菌性、滅菌、免疫隔離、血液－細胞分離、雑菌汚染防止)
Scalability(大きさの可変性)	Ease of scale up, reactor size, high blood flow rate, high medium circulation rate(スケールアップの容易さ、リアクターの大きさ、高血流速度、高灌流速度)
Good environment for cell growth(良好な細胞環境)	High cell density, high viability, oxygen transport, nutrient transport, mass transfer(高細胞濃度、高生残率、酸素移動、栄養供給、物質移動)
Mimicking native liver functions(肝機能模倣)	3-dimensional structure, suitable scaffold, spheroid formation and maintenance, co-culture of hepatocytes with nonparenchymal liver cells(3次元構造、最適な足場、スフェロイドの形成と維持、肝実質細胞と非実質細胞の共存)
Handling(扱いやすさ)	user-friendly, short preparation time, ease of setup, ease of control and operation, maintenance of reactor with cells, cost, availability(使いやすさ、準備時間の短さ、セットアップの行いやすさ、制御・操作の容易さ、コスト、手に入りやすさ)

アクターを選定することができる（図3）。評価項目については色々と議論があるが、ここでは、我々は、人工肝に必要な評価項目として下記の表3に示す5項目を選択し、これらについて一対比較を行い、ランク付けをした[9]。

この評価項目について、3人のパネリスト（生物化学工学者、材料科学者、医科学者）によってAHP手法を用いて9段階の評価を行った。また、最終的に評価するバイオ人工肝リアクターとしては、下記の代表的な6種類を選択した。

(1) Collagen entrapped hollow fiber（コラーゲン固定化ホロファイバー）(Hu *et al.* 1997)
(2) Capillary network hollow fiber（Modular Extracorporeal Liver Support (MELS)）(多重ホロファイバー型) (Gerlach *et al.* 2002)
(3) Radial flow bioreactor（ラジアルフロー型）(Matsuura *et al.* 1998)
(4) Rigid-type multi-capillary polyether polyurethane foam (MC-PUF) hepatocyte spheroid packed-bed module (MC-PUF/スフェロイド充填槽型) (Funatsu *et al.* 2001)
(5) Packed-bed type reactor using reticulated polyvinyl formal (PVF) resin as a supporting material（多孔質充填槽型）(Ohshima *et al.* 1997)
(6) Circulatory flow bioreactor with glass fiber cloth, "Cygnus"（回流式培養装置）(Enosawa *et al.* 2000)

次に、評価項目それぞれについて各リアクター間の一対比較を行った。本手法では、回答後に、論理矛盾について判定し、回答に論理矛盾が無い場合についてのみ回答となる。これは人間が回答するために、回答の中には論理矛盾が生じている場合があるためである。論理矛盾とは、例えばA、B、Cの3種類のリアクターを比較する際に、もしA>B、B>C、C>Aという回答が得られたとすると、A、B、Cの3つのリアクター間でランク付けすることができない場合を指す。比較すべき対象の数が少ない場合には論理矛盾は生じにくいが、多数の場合は、意図せずして生じることがある。本比較でも、3人の評価パネリストのうち、1名の回答には論理矛盾が生じたため、論理矛盾の回答は取り除いて最終評価を行った。

図4に示されるように、どのパネリストも、安全性を最重要項目においているが、その比重のかけ方は異なる。また、データとしては示していないが、評価したリアクターに関しても、リアクターごとの重要度の違いが明らかになり、各リアクターに欠けているとパネリストが考えている項目および総合的に適しているリアクターが選択可能である。本数値は絶対的なものでは無く、あくまでも人間（ここでは、各々専門の異なる3名のパネリスト）の相対的な判断を数値化したものであり、パネリストの選択に左右される面があるのは否めない。さらに多数のバックグラウンドの異なるパネリストを集め、各項目についてAHPを用いて比較することで、様々な研究者が開発したバイオ人工肝システムの相互かつ総合評価が可能になると考えられる。

4. さいごに

バイオ人工肝を構成する要素としての細胞や装置、材料の開発は一時期の停滞を抜け出して、近年発展めざましいものがある。一方、バイオ人工肝自体に求められる性能や評価については、依然として定まっておらず、開発された新技術の目指すべき方向性はあまりはっきりしていない。方向性について、関連する研究者全体で討論すべき時期に来ているのではないだろうか。

謝辞：本研究のバイオ人工肝のクリアランスを用いた評価に関しては、国立成育医療センター研究所移植・外科研究部絵野沢伸先生と、AHPを用いた評価に関しては大阪大学大学院薬学研究科川瀬雅也先生との共同研究である。両先生に感謝申しあげます。

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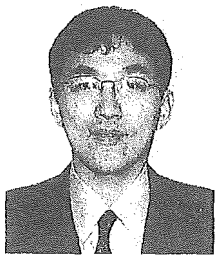
総合論文

動物細胞培養による物質生産系構築とティッシュエンジニアリングへの応用

（平成16年度　日本生物工学会照井賞受賞）

大政　健史



Biochemical Engineering in Cell and Tissue Engineering

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The application of mammalian cell cultivation is a great achievement of biotechnology. There are two categories of application: use of the mammalian cell as a production host for biologics; and use of the mammalian cell itself as the end product. In the present article, we review construction methods for suitable gene-amplified cell lines and glycosylation control as examples of biologics production; and a bioartificial liver support system as an example of cell product.

[**Key words:** mammalian cell, tissue engineering, gene amplification, glycosylation, artificial organ]

はじめに

動物細胞の産業利用は，細胞を生体外に取り出し，これを培養する手法の開発が出発点となっている．19世紀後半から，動物の組織から細胞を取り出すことはなされていたが，実際に動物細胞を *in vitro* で培養する技術は1907年にHarrisonによって行われたオタマジャクシの脊索から取り出した細胞を培養する実験にて本格的に始められ，[1] 約100年弱の歴史しかない．

細胞の産業応用は，1950年代の合成培地の開発を受けて，さらに1970年代後半から活発に研究される無血清培地の開発に後押しされた形で，インターフェロンやエリスロポエチンのような生体内に微量しか存在しない物質の生産手段として進められた．これと同時に高密度灌流培養の技術開発，遺伝子増幅をはじめとする高発現ベクターの開発も行われ，動物細胞の産業利用が本格化した．さらに1990年代に入ると，ティッシュエンジニアリング，再生医工学の発展に伴い，細胞自身を治療として用いる手法が開発されるようになってきた．

現在，細胞の産業応用は大きく2つに分類可能であると言える．一つは動物細胞の生産する物質を利用する，すなわち生産の場としての利用法（バイオ医薬品生産，ワクチン生産など），もう一つは細胞自身を利用する手法（バイオ人工臓器，再生医療，細胞を用いた評価系など）である．現在，前者に関しては，エリスロポエチン，顆粒球コロニー刺激因子（G-CSF），抗体医薬に代表されるバイオ医薬品の大規模生産が行われ，これからさらなる成長が見込まれている．一方，後者に関しては，現在産業化を目指した応用のための基礎研究が活発に行われており，今後の発展が期待されている．[2–4] 本総合論文においては，筆者らのこれまで行ってきた動物細胞による物質生産系の構築から始まり，これを応用したバイオ人工肝臓への応用までについて述べ，動物細胞の産業応用について考察する．

1. 細胞株構築プロセス

一般に動物細胞による物質生産は，微生物と比較して生産性が低く，培養にもコストがかかる．たとえば，

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