

In Vivo Estimation of Bioartificial Liver With Recombinant HepG2 Cells Using Pigs With Ischemic Liver Failure

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Biological efficacy of a recombinant human hepatic cell line, glutamine synthetase transfected HepG2 (GS-HepG2), was examined with large-scale culture in a circulatory flow bioreactor and in pigs with ischemic liver failure. GS-HepG2 cells were cultured in a circulatory flow bioreactor from 5×10^7 to 4×10^9 cells for 109 days. The cells showed ammonia removal activity even under substrate (glutamic acid)-free medium, suggesting that the GS catalyzed the activity using intracellular glutamic acid that had been pooled during conventional culture. When GS-HepG2 bioartificial liver (BAL) was applied to pigs with ischemic liver failure, survival time was prolonged to 18.8 ± 6.1 h (mean \pm SD, $n = 4$) from 13.8 ± 5.4 h ($n = 6$) and 10.7 ± 4.1 h ($n = 6$) (groups treated with cell-free BAL and treated with plasma exchange and continuous hemodiafiltration, respectively). Laboratory data indicated the tendency for improvement in increase of blood ammonia level and decline of blood coagulation indices in the GS-HepG2 BAL-treated group. The advantages and potential for the cell line as a bioreactor in BAL is also discussed, comparing to those of isolated porcine hepatocytes.

Key words: Ammonia removal; Glutamine synthetase; HepG2; Bioartificial liver

INTRODUCTION

A bioartificial liver (BAL) support system is currently expected to be a novel therapeutic device for the end stage of hepatic failure (2,3,8,11). BAL treatment will improve a recipient's condition to bridge to a successful transplantation and will support the critical stage of the postoperative period. There are mainly two options for cells used in the bioreactor: isolated hepatocytes (2,3,8) and lined cells (11). The former, usually isolated porcine hepatocytes, are multifunctional and of high performance, but the functioning time is relatively short (approximately up to 10 days), secreted proteins as well as cell components possess xenoantigenicity, and cell processing such as isolation, inoculation, and culture is considerably laborious. Moreover, risk of zoonosis is a current concern from not only a clinical but also from a public standpoint (1,10). On the other hand, while hepatic cell lines are advantageous in supply and handling procedures, cell lines almost lose their original functions (5) and, from functional evaluation, cell lines are regarded to be unsuitable for BAL (12).

However, cell lines have potential usefulness, especially from an industrial view point, such as supply, storage, quality control, etc. To compensate for the diminished activity, we used recombinant technology. Glutamine synthetase (GS) gene was transfected to human hepatoblastoma cell line, HepG2, and GS-HepG2 was established. The ammonia removal activity of GS-HepG2 was approximately 1/7–8 of that of isolated porcine hepatocytes (4), whereas the original HepG2 cells showed no ammonia removal activity. In this communication, we demonstrate the in vivo estimation of bioartificial liver with GS-HepG2 cells using pigs with ischemic liver failure and discuss the advantages and prospects of this cell line as a bioreactor in BAL.

MATERIALS AND METHODS

Cells and Culture Condition

GS-HepG2 cells were developed and cultured as described previously (4). Briefly, the glutamine synthetase gene from Chinese hamster ovary cells was inserted in pBR322 plasmid with cytomegalovirus promoter and transfected into HepG2 cells by electroporation. Cloning

and amplification of the GS gene was performed by culturing cells under the presence of methionine sulfoximine. GS-HepG2 cells thus obtained were cultured in glutamine-free RDF medium containing glutamate and 5% fetal bovine serum in the circulatory flow bioreactor. The initial number of inoculated cells in the bioreactor was 5×10^7 cells and cells were grown to 4×10^9 cells. The bioreactor was kept at 37°C and fresh medium, air, CO₂, and oxygen were supplied as described previously (4).

Ischemic Hepatic Failure Model

Domestic piglets (2.5–3.5 months old), weighing 20–27 kg, were used for in vivo estimation of BAL. The operating procedure of ischemic liver failure and BAL treatment was performed as described previously (9). Briefly, the piglet was anesthetized and maintained by 0.5–1.5% Isoflurane (Abbott, Illinois, USA) with muscle relaxant (pancuronium bromide, 2 ml/h, IV). Monitoring catheters were inserted to the iliac artery and vein to measure the arterial and central venous pressure. A double lumen catheter was placed in the right jugular vein for blood access of extracorporeal BAL circulation. To establish a portosystemic shunt, outlet and inlet catheters were put in the portal vein and left jugular vein, respectively, and portal blood was bypassed using a centrifugal blood pump (HPM-15, Nikkiso, Tokyo, Japan). Then the portal vein, hepatic artery, and common bile duct were ligated. Pigs were supplied inorganic salt solution (Sublood B, Fusou Pharmaceuticals, Osaka, Japan) at a rate of 10 ml/kg/h during the operation and, after the operation, the solution was changed to Sublood B containing 5% glucose at a flow rate of 4 ml/kg/h. No vasopressor was used throughout the experiment.

Treatment of BAL and Plasma Exchange and Continuous Hemodiafiltration

Three hours after the completion of total liver ischemia, BAL treatment was started (9). Blood taken from the double lumen catheter in the right jugular vein was led at a rate of 15 ml/min to a plasma exchange column (PP-03, Ube Medical, Tokyo, Japan), using hemodialysis console (JUN-500, Ube Medical, Tokyo, Japan). Separated plasma (3 ml/min) was flown into the circulatory flow bioreactor with or without GS-HepG2 cells (treated group and control group, respectively) and returned to the jugular vein via the double lumen catheter. All extracorporeal space (550 ml) was primed with Sublood B and pigs were transfused with 600 ml of fresh frozen pig plasma from the beginning of the BAL treatment for 3 h (167 ml/h). Plasma exchange (PE) and continuous hemodiafiltration (CHDF) were performed with specified columns (PP-03, Ube Medical, Tokyo, Japan and FH-66D CAVH filter, Gambro, Germany) using

two hemodialysis consoles, which were tandemly connected.

Monitor of Physiological Indices

Heart rate, arterial and central venous pressure, and intracranial pressure were monitored continuously (Model 66S, Hewlett Packard, USA). Blood gas, hemoglobin, electrolyte, and laboratory data [glucose, ammonia, aminotransferases, coagulation indices (activated clotting time (ACT), hepaplastin test (HPT), activated partial thromboplastin test (APTT)] were estimated every 3 h after the completion of ischemic liver failure (9). Termination of the heartbeat was regarded as the end point of survival.

RESULTS

Ammonia Removal Activity of GS-HepG2 in the Various Conditions

Ammonia removal activity was detectable in not only normal but also substrate (glutamic acid)-free culture medium (Fig. 1). Ammonia removal rates were calculated by the approximation lines to be almost the same in the two conditions (159.3 and 141.7 $\mu\text{mol/L/h}$, respectively). Reflecting the decrease of ammonia, glutamine in the culture medium was increased in both conditions (data not shown). GS-HepG2 cells also decreased ammonia concentration in plasma from a hepatic failure pig (ammonia concentration 3.0 mmol/L) with the de-

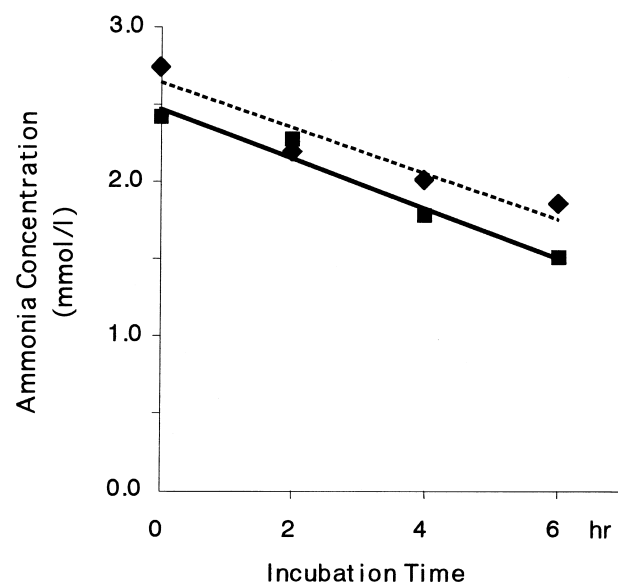


Figure 1. Metabolic activity of GS-HepG2 cells in various culture conditions. GS-HepG2 cells (approximately 2×10^9 cells) were cultured in a circulatory flow bioreactor. During experiment, culture medium used was as follows: normal RDF medium (■), RDF medium without glutamic acid (◆).

crease rate of 187.5 $\mu\text{mol/L/h}$, which was comparable to the activity in the culture medium (data not shown).

In Vivo Estimation of the Efficacy of GS-HepG2 BAL Using Pigs With Ischemic Liver Failure

GS-HepG2 cells were grown from 5×10^7 to 4×10^9 cells during the culture period (109 days). Judging from the glucose consumption rate, the cells became stationary from culture day 60, and the BAL treatment experiment was done with the bioreactor four times at this stage. No bacterial contamination occurred during the repeated use of BAL for 6 weeks. After the *in vivo* treatment, the cell damage by the treatment was likely to be insignificant because ammonia removal activity was not changed before and after the experiment (data not shown).

Survival times of the pigs treated with cell-free BAL, GS-HepG2 BAL, and PE and CHDF were 13.8 ± 5.4 h ($n = 6$), 18.8 ± 6.1 h ($n = 6$), and 10.7 ± 4.1 h ($n = 6$), respectively (Table 1). Although there was no statistical significance among these three groups, the mean survival time of the GS-HepG2 BAL-treated group was the longest. Increase of blood ammonia tended to be lower in GS-HepG2 BAL-treated group than in cell-free BAL- and PE and CHDF-treated groups from 15 h after the hepatic ischemia (Fig. 2A). Physiological data also indicated improvement of coagulation indices, such as ACT, HPT, and APTT (Fig. 2B, C, D). ACT and APTT were kept in the physiological range during the experiment (Fig. 2B, D).

DISCUSSION

Development of BAL is now entering clinical trials (3,8,11). Except for the C3A/HepG2 cell line (11), most researchers use porcine hepatocytes (2,3,8). Although porcine hepatocytes possess efficient physiological functions, isolated hepatocytes survive for 1 week at most. Moreover, recent reports pointed out the risk of porcine endogenous retroviral infection (1,10). On the other hand, immortalized cell lines have powerful growth activity and, for instance, some cell lines can be maintained under serum-free medium. This growth ability will provide the possibility of repeated use of the bioreactor after the exposure of plasma from a patient with

fulminant hepatic failure. Although the interval of repeated use was 7–14 days in this experiment, the interval can be shortened if other back-up modules are available.

GS-HepG2 cells exhibited ammonia removal activity in substrate-depleted condition and porcine plasma from a pig with ischemic hepatic failure. In hepatic failure plasma, glutamine concentration was increased, probably due to systemic tissue damage, and, under such severe condition, GS-HepG2 catalyzed ammonia removal. GS requires ammonia, glutamic acid, and ATP as substrates and cofactors (6). In the substrate-depleted condition, perhaps GS catalyzed the reaction with the intracellular pool of the substrates, which had been supplied during normal culture medium. The reaction cofactor, ATP, was likely to be produced by the cell itself. Because ammonia removal activity under various circumstances was almost the same, GS used the intracellular pool, preferably to substances in the outside medium or plasma.

Using GS-HepG2 cells, the BAL treatment prolonged mean survival time compared with that of cell-free BAL-treated and PE and CHDF-treated groups, although the data were not statistically significant. At present, we have no clear explanation about the prolongation. However, besides survival time, other vital signs (e.g., body movement) seemed to be improved. One possible reason may be removal of toxic cytokines as reported previously (4). Unexpectedly, blood coagulation indices were improved in the GS-HepG2 BAL group. Because only a small amount of plasma protein was produced by GS-HepG2 cells (data not shown), this effect may be due to the suppression of consumption of coagulation factors by the contact of plasma with GS-HepG2. Although the mechanism remains to be clarified, the improvement of the indices is correlated with the prolongation of survival time because coagulation disorder is one of main causes of death in fulminant hepatic failure.

Isolated porcine hepatocytes have been recognized as most suitable for bioreactor cells in BAL. As shown in Table 2, isolated hepatocytes exhibit highly differentiated functions, and recent development of culture machinery and cell matrix actualizes equivalent performance to physiological activities (7,13). However, the

Table 1. Survival Time of Pigs With Ischemic Liver Failure Under Different Conditions

Condition*	Survival Times (h)	Mean \pm SD (n)
Cell-free BAL	9.0, 10.1, 11.1, 11.3, 20.6, 20.7	13.8 ± 5.4 (6)
GS-HepG2 BAL	12.1, 16.2, 20.6, 26.3	18.8 ± 6.1 (4)
PE & CHDF	6.6, 7.2, 7.9, 11.0, 15.4, 15.8	10.7 ± 4.1 (6)

*Pigs were treated with cell-free circulatory flow bioreactor, the reactor containing GS-HepG2, and plasma exchange and continuous hemodiafiltration (PE & CHDF).

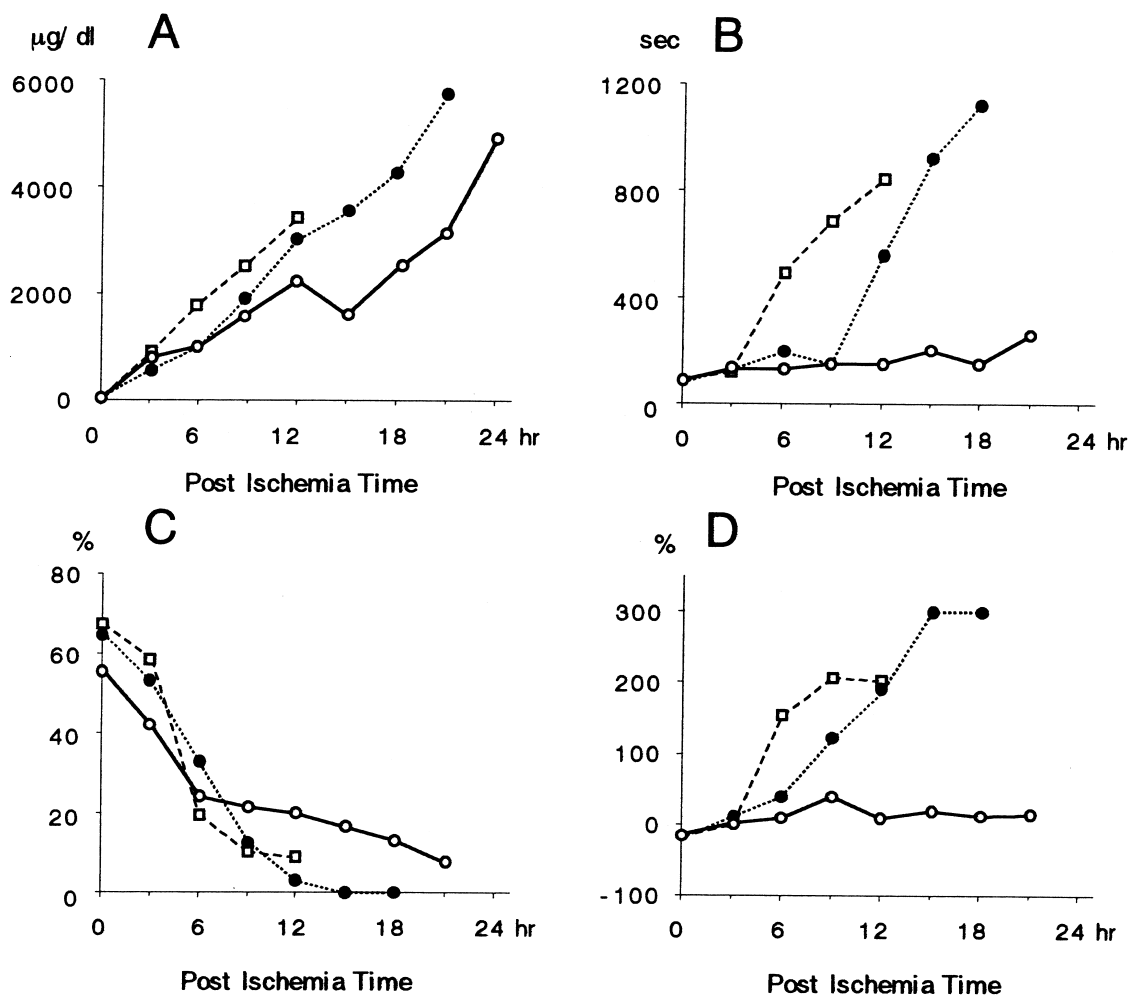


Figure 2. Time course changes in laboratory data on pigs with ischemic hepatic failure. The mean values of blood ammonia (A), activated clotting time (ACT) (B), hepaplastin test (HPT) (C), and activated partial thromboplastin time (APTT) (D) of the pigs with the treatment of cell free-BAL (\diamond , $n = 6$), GS-HepG2 BAL (\circ , $n = 4$), and plasma exchange and continuous hemodiafiltration (\bullet , $n = 6$) are shown.

Table 2. Comparison of Cell Lines and Isolated Hepatocytes as Bioreactor Cells for Bioartificial Liver

	Cell Lines	Isolated Hepatocytes
Function	low, restricted	high, differentiated
Culture time	long (~several months)	short (~10 days)
Advantages	quality control; stock reserve; repeat use for a single patient	high performance
Problems	oncogene; low function	xenozoonosis; xenoantigenicity; laborious manufacturing
Solution	risk assessment of oncogene; recombinant technology	risk assessment of xenozoonosis; immunoisolation; cryopreservation; use of human hepatocytes
Ideal solution	use of human hepatic stem cells	

present assembly procedure of BAL using primary culture hepatocytes seems to be intrinsically incompatible to industrial production of medical equipment, due to the laborious and handwork process of cell isolation and inoculation, which hinders quality control and stock reserve. Although there is limited clinical evidence, porcine hepatocytes and their secretions have xenoantigenicity in humans, and it is equivocal whether porcine hepatocytes assist synthesis of serum proteins that were depleted by hepatic failure. Moreover, the presence of xenozoonosis, such as porcine endogenous retrovirus, is considered to be potentially hazardous not only to patients themselves but also to public health (1). To solve the above problems and to utilize porcine hepatocytes, well-balanced immunoisolation, risk assessment of xenozoonosis, and further development of cryopreservation are indispensable. Another option to develop BAL with primary culture hepatocytes will be use of human hepatocytes instead of xenogenic hepatocytes. However, development of cryopreservation will be needed to use human hepatocytes for BAL. On the other hand, cell lines have almost reciprocal advantages and disadvantages for use in BAL, compared with porcine hepatocytes. Among the points described above, cell lines are superior in supply and culture time, leading facility of quality control and stock reserve. Low function must be compensated by recombinant technology, as has been already applied for the pharmaceutical production of bioactive substances such as interferon, tissue plasminogen activator, insulin, etc. At the same time, the strategy to certify that those pharmaceuticals are free from viral particles and oncogenes will assist the risk assessment of cell lines for BAL. Although we used an established cell line, HepG2, in this study, if safer artificial cell lines such as telomerase transduced cells are available, we will develop BAL with the cells on the basis of our present technology. In this sense, human hepatic stem cells will be the final and ideal solution for bioreactor cells.

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