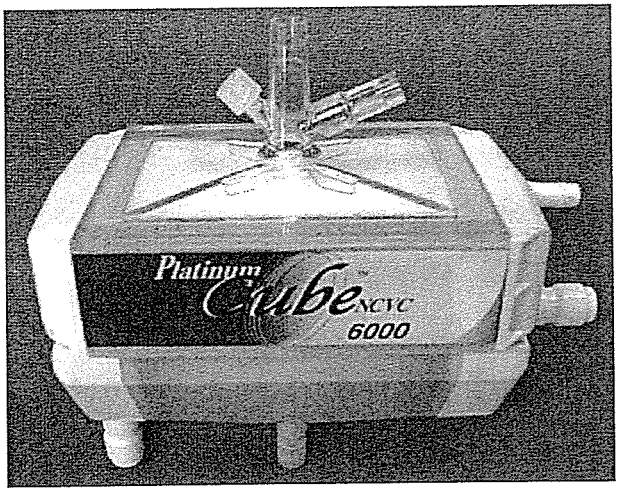
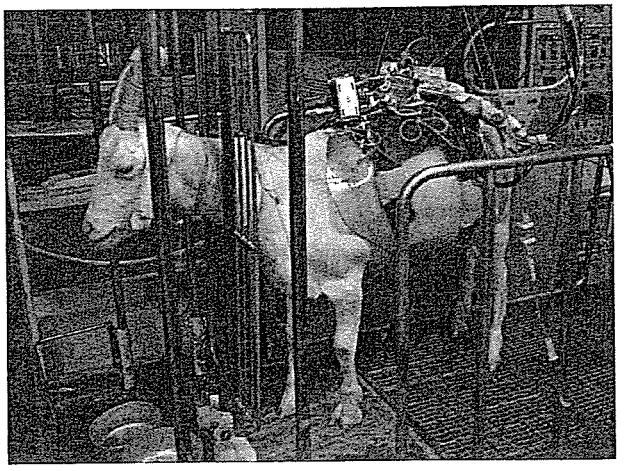


図❻ T-NCVC コーティング膜型人工肺 Platinum Cube NCVC 6000



図❼ T-NCVC コーティング PCPS システムの長期動物実験風景

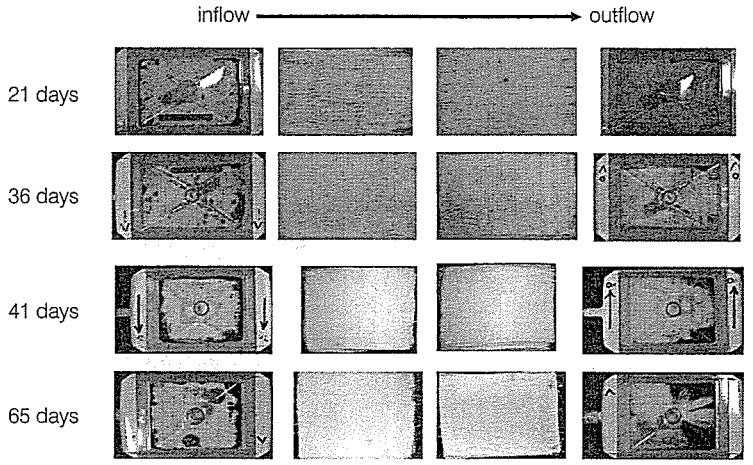
れた．また，T-NCVC コーティング Toyobo-NCVC VAS は，2005（平成 17）年に厚生労働省より医療機器として承認を受け，今後臨床使用が開始される見込みであり，補助時人工心臓装着患者の臨床成績の向上に貢献できることが期待されている．

III．T-NCVC コーティング膜型人工肺 Platinum Cube NCVC シリーズ

膜型人工肺を用いた経皮的心肺補助法（percutaneous cardiopulmonary support：PCPS）や ECMO（extracorporeal membrane oxygenation）は，重症呼吸循環不全患者への強力な治療法として，心臓外科領域に限らず，救命救急領域などにおいても広く普及しはじめている．しかしながら，これら新しい救命法や治療法が普及するにつれ，その長期使用や適用の拡大などに対するハードウェア整備の立ち後れが指摘されるようになってきた．

かかる問題に対し，われわれは，大日本インキ株式会社と共同開発をおこなってきた中空糸表面に 200 nm 以下の緻密層を形成し，ガス交換能にもすぐれた特殊ポリオレフィン膜を使用した膜型人工肺に，さらに T-NCVC コーティングを採用し，長期の耐久性および生体適合性にすぐれた次世代型膜型人工肺 Platinum Cube NCVC シリーズを完成させた（図❻）．また，この膜型人工肺 Platinum Cube NCVC 6000 を使用し，回路全体に T-NCVC コーティングを施した次世代型 PCPS システムを開発し，成ヤギを用いた長期動物試験により，本システムの血液適合性および長期耐久性について検討をおこなった．

実験では成ヤギに対し，右心房脱血，頸動脈送血による静動脈バイパス術を施し，覚醒下において試験システムを最長 65 日間持続的に駆動させ，その血行動態，血液，血球成分の変化，および実験終了後におけるシステム内での血栓形成状況について検討をおこなった（図❼）．なお，実験期間中の抗凝血療法はヘパリンを手術中のみ使用し，術後の長期管理中には血圧モニター用ライン維持を目的としたきわめて微量な投与をおこなうのみで実験をおこなった．その結果，全例において実験期間中には，膜型人工肺のガス交換能などの機能低下や血漿リークなど異常所見は認められず，良好な状態で静動脈バイパスを維持することが可能であった．血液生化学的には，実験期間中の賦活化血液凝固時間（activated clotting time：ACT）は，術前値とほぼ変化せず，血小板数の軽度の低下を認めたのみで，アンチトロンビンIII，フィブリノーゲンおよびフィブリノーゲン・フィブリン分解産物も最長 2ヵ月まで著変は認められなかった．実験終了後の膜型人工肺の血栓形成状況は，そのケース辺縁部に若干の血栓形成を認めたが，中空糸膜の層間内部にはほ



図❽ 長期動物実験後の T-NCVC コーティング膜型人工肺
抗凝血療法をおこなわない長期動物実験後においても，本人工肺にはケーシング周辺に血栓形成が認められのみであり，中空糸膜には血栓の付着などは認められなかった．
（西中知博ら，2002[4]より引用）

とんど認められなかった（図❽）．

以上より，われわれの開発した次世代型膜型人工肺 Platinum Cube NCVC シリーズを組み入れ，回路全体に T-NCVC コーティングを施した PCPS システムは，抗凝血療法を不要とする持続的な長期的使用が可能であることが示された．また，本システムは長期に安定した血液適合性能を維持しうることから，従来のシステムでは適応できなかった，出血を伴う外傷や脳血管障害および長期心肺補助を必要とする種々の症例への適応拡大が見込まれ，従来の静動脈バイパスのみならず，低流量循環が必要な静脈―静脈および右房―肺動脈バイパスなどのアプローチ選択の多様化など，さまざまな病態ごとに適した循環および呼吸補助法に対応可能な次世代治療に適したシステムになりうると考えられた．

さらに，われわれは現在 Platinum Cube NCVC シリーズのなかでも最小モデルである Platinum Cube NCVC 2000 を組み入れた低灌流用 PCPS システムの開発をおこなっており，小児用あるいは新生児用 ECMO に最適なシステムの開発，製品化が期待されている[7]．

■ おわりに：今後の展望について

重症心不全ないし呼吸不全患者に対する人工臓器治療の適用数は，機器やその材料の発達により安全性や耐久性が向上し，年々増加傾向にある．また，傷害臓器の回復や移植（Bridge to Transplantation）を見据えた比較的短期の使用を目的とした適用から，ドナー不足による移植待機時間の延長や，心機能の回復を見込めない症例に対する恒久的な使用を目的とした Destination Therapy への適用のための長期治療まで，その使用法は多岐にわたる傾向にある．かかる状況に対し，人工臓器の長期的な耐久性，および確かな安全性の向上を求める声は根強く，とくに血液との接触が不可避な循環器系人工臓器においては，長期使用の安全性を確保するため，抗血栓性や生体適合性の長期的な維持が重要な課題となっている．

われわれが開発した表面処理技術 T-NCVC コーティングは，ナノサーフェーステクノロジーを応用した新しいコンセプトに基づき，きわめて高い血液適合性と同時に長期耐久性や処理の簡便性を実現することに成功した新規技術である．これらの技術を統合して開発する，抗凝血療法不要の長期連続使用が可能な次世代型人工心臓や膜型人工肺は，従来の機器の最大の欠点であった血栓塞栓症の発生や抗凝血療法併用による致死性出血性臓器障害の発生を激減させ，外傷患者，脳血管障害既往患者および従来システムでは適応になりえなかった慢性呼吸循環不全患者の急性増悪時の短中期心肺補助に対する中長期補助も可能とするなど，その特性を十分に活用させた適用範囲，適用環境が飛躍的に拡大することが期待さ

れている．現在の研究では，T-NCVC コーティングが，器材表面に接触する細胞の接着作用を著しく阻害する効果を有することが見出されているものの，この細胞接着阻害作用は，従来提唱されていた作用機序とは異なる結果が多く認められていた．故に，T-NCVC コーティングの有する細胞接着抑制機序には，従来の抗血液凝固性機序とは異なった新しい機序が存在する可能性が想起され，かかる新機序を解明することにより T-NCVC コーティングの血液に対する適合性をより機能的に向上させることが可能になると考えられる．

また，本コーティングに使用されるヘパリンは，生物製剤として人畜共通感染症に対するスクリーニングの必要性があり，その供給についても不安定な側面をもつことが問題として挙げられる．そのため，これからの次世代型の抗血栓法の開発には，T-NCVC コーティングの機能を損なわず，ヘパリン剤を合成高分子化合物に置き換えることが必要であり，かかる諸問題を早期に解決することは，さらに次世代型の生体適合性表面処理法を開発するための重要な課題と考えられる．

しかしながら，現段階において非常に優秀な成績を得ている T-NCVC コーティングをもってしても，人工臓器内に発生する血栓形成を完全に抑制することは非常に困難であり，Toyobo-NCVC VAS では，弁輪周囲部のように血栓形成に対し構造的に不利な部位への適用には限界があることも事実である．したがって，これからの人工臓器開発においては，ナノ単位で表面改変が可能なナノサーフェーステクノロジーと，構造学的および流体工学的な見地からマクロ構造を改良するマクロサーフェーステクノロジーを同時に見直すことが重要であり，ナノからマクロへ，マクロからナノへの技術の循環が，より安全ですぐれた次世代型デバイス開発に必要であると考える．

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The Significant Improvement of Survival Times and Pathological Parameters by Bioartificial Liver With Recombinant HepG2 in Porcine Liver Failure Model

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We developed a bioartificial liver (BAL) containing human hepatoblastoma cell line, HepG2, with the addition of ammonia removal activity by transfecting a glutamine synthetase (GS) gene and estimated the efficacy using pigs with ischemic liver failure. GS-HepG2 cells showed 15% ammonia removal activity of porcine hepatocytes, while unmodified HepG2 had no such activity. The established GS-HepG2 cells were grown in a circulatory flow bioreactor to 3.5–4.1 × 10^9 cells. Survival time of the animals treated with GS-HepG2 BAL was significantly prolonged compared to the cell-free control (14.52 ± 5.24 h vs. 8.53 ± 2.52 h) and the group treated with the BAL consisting of unmodified wild-type HepG2 (9.58 ± 4.52 h). Comparison showed the cell-containing BAL groups to have significantly fewer incidences of increased brain pressure. Thus, the GS-HepG2 BAL treatment resulted in a significant improvement of survival time and pathological parameters in pigs with ischemic liver failure.

Key words: Bioartificial liver (BAL); HepG2; Ischemic liver failure; Porcine hepatocytes

INTRODUCTION

The bioartificial liver (BAL) was conceived as a support system providing liver function by using an artificial module with living hepatic parenchymal cells (2,4, 15). Most researchers use primary hepatocytes from pigs as the reactor cells, because 1) primary culture hepatocytes have a full range of hepatic functions, 2) porcine hepatocytes are much easier to obtain practically and ethically than human primary hepatocytes, and 3) porcine materials have already been utilized for several medical purposes, although their clinical use is still adventurous (16,20,24). However, the hepatic functions such as drug metabolism and ammonia removal are reduced under the culture condition, and BAL activity only lasts for 10–20 days. When hepatocytes have been cryopreserved, cell viability and functions, especially cell attachment to the culture matrix, markedly decrease. In addition, from a public health viewpoint, the potential risk of infection from porcine endogenous retrovirus applies not only to patients themselves, but also the public at large (21). Therefore, the BAL with primary porcine hepatocytes is not yet suitable as a medical apparatus, even under conditions of well-defined quality control.

When lined cells are used as bioreactor cells, the major problem is their decreased hepatic functions (7). Nevertheless, quality control is easier with these cells than with primary hepatocytes. In addition, advantages in use of lined cells are as follows: 1) indefinite growth, 2) feasibility of frozen storage and elimination of infectious agents, 3) a low ethical problem for cell preparation. The human hepatoblastoma cell line, HepG2, is one promising cell line for the bioreactor of the BAL because HepG2 maintain differentiated characteristics of normal parenchymal cells, such as drug-metabolizing activity and production of albumin and transferrin [(6) and our unpublished results]. Previously, the HepG2 subline, C3A, was examined for effectiveness as reactor cells of BAL or ELAD® (extracorporeal liver assist device) in an acetaminophen-intoxicated canine model and a clinical study was performed (5,22). Recently, the development of ELAD®, including phase I and II clinical study, was transferred to Vital Therapies, Co. Ltd (San Diego, CA). However, there was an unresolved problem,

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in that neither HepG2 nor C3A had ammonia removal activity (6).

We have added ammonia removal activity to HepG2 by transfecting the glutamine synthetase (GS) gene (9). GS is localized in hepatic parenchymal cells aligned around the central vein and catalyzes an accessory pathway of ammonia removal by synthesizing glutamine from glutamate and ammonia. We have previously shown that GS-transfected HepG2 (GS-HepG2) removed ammonia in the culture medium with 1/7 of the activity of porcine hepatocytes (9,19). In this report, we applied the BAL with GS-HepG2 to pigs with ischemic liver failure, to observe the survival time and other parameters related to hepatic function. Our standardized simple model of liver failure using pigs enabled a large number of experiments with highly reproducible results (18). We also configured a novel criterion in survival time, active survival time in the previous report (18). Active survival time is defined as the time until when the systolic pressure becomes less than 50 mmHg, while gross survival time is until the cessation of heart beat. In this report, we analyzed all survival data with the concept of active survival time. These two methodological improvements enabled the comparison of various experimental conditions, including the present conventional treatment of plasma pheresis, resulting in the discovery of the significant prolongation of survival time with the recombinant HepG2 BAL treatment and decreased incidence of brain pressure rise.

MATERIALS AND METHODS

Bioreactor Cells and Culture System

We have previously described the method for establishing the bioreactor cells, GS-HepG2 (9,19). Briefly, the hamster GS gene was transfected to HepG2 (RIKEN Cell Bank, Tsukuba, Ibaraki, Japan). The transfected cells were cultured in the presence of methionine sulfoximine to amplify the transfected GS gene. The GS-HepG2 (2×10^8 cells) that were subcultured in 20 culture flasks (150 cm^2, 430824, Corning, NY, USA) were inoculated into the circulatory flow bioreactor (U.S. Patent 5270207, 1993) and cultured as shown in Figure 1. When the cells grew to stationary phase (total cell number; $3.5–4.1 \times 10^9$) as indicated by the glucose consumption rate, in vivo estimation of the BAL was carried out. As one of the control experiments, a BAL with wild-type HepG2 cells was prepared, as mentioned above, for the GS-HepG2 BAL. A BAL with one batch of reactor culture was used for a series of four in vivo experiments at 1-week intervals. Immediately after finishing the series, the cells were harvested from the culture matrix to determine cell number and viability, using crystal violet and trypan blue solution (9).

Animals and Hepatic Failure Model

Domestic piglets cross-bred with Large-Yorkshire, Landrace, and Dulroc (approximately 25 kg, 2–3 months old) were purchased from Kidokoro farm (Atsugi, Kanagawa, Japan) and kept in our animal facility prior to the operation. The animals were used within 2 days of transportation. All experimental procedures were performed according to the institutional animal ethics guidelines, based on the guidelines of the National Institute of Health, USA (23). Ischemic liver failure surgical procedure has previously been described in detail (18). Briefly, after the portal flow was by-passed to the jugular vein with a centrifugal blood pump (HPM-15, Nikkisou, Tokyo, Japan), the portal vein, common hepatic artery, and common bile duct were ligated. Inorganic salt solution (Sublood B, Fusou Pharmaceuticals, Osaka, Japan) containing 5% glucose was continuously supplied after the operation. The concentration of inhalation anesthetic, tidal volume, oxygen concentration, the rate of infusion, and the addition of glucose, heparin, and sodium bicarbonate were adjusted as expedient, in accordance with our previous report (18). No vasopressor was used in the experiment.

Treatment With BAL and Plasma Pheresis

Three hours after total liver ischemia, the BAL treatment was started, as described in Figure 1. Blood access was performed using a double lumen catheter (14 gauge, Medicut UK-II Catheter Kit®, TYCO Healthcare, Mansfield, MA, USA) inserted into the right atrium via the right jugular vein. Outflow of blood was led to a hemodialysis console (JUN-500, Ube Medical, Tokyo, Japan) at 15 ml/min, and plasma was separated at a rate of 3 ml/min by hollow fiber column (PP-03, Ube Medical, Tokyo, Japan). The plasma was passed through the BAL with or without reactor cells and returned to extracorporeal circulation. Extracorporeal volume (200 ml in the tube and 550 ml in the bioreactor including hollow fiber ventilation unit) was primed with Sublood B. During the first 3 h from the beginning of BAL treatment, the animals were transfused with 500 ml of freeze-stored pig plasma at a rate of 167 ml/h.

In the group treated with plasma pheresis, we connected two hemodialysis consoles serially: one for plasma separation and the other for hemodiafiltration (12). The plasma separation flow rate was the same as described above. The supplementation with normal pig plasma was done at an equivalent rate during the first 6 h. Total amount of exchanged plasma was approximately 1000 ml, almost equal to the total body plasma of a piglet. Hemodiafiltration was performed with the second console, using hollow fiber column (FH-66D, Gambro, Hechingen, Germany). Flow rates were 15 ml/min for blood, 100 ml/min for dialysis fluid, 150 ml/min for fil-

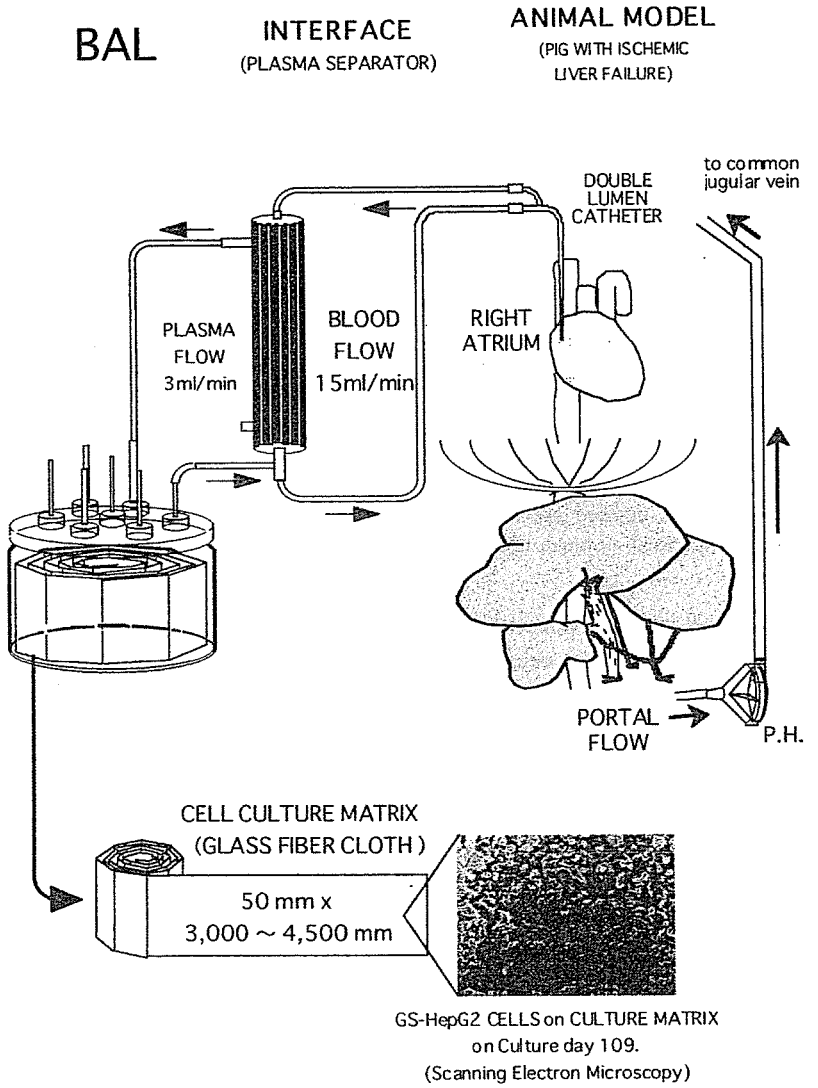

Figure 1. Schematic illustration of connection of BAL and hepatic failure pig. Separated plasma was flown into the BAL. Separated plasma was flown into the BAL and returned to blood flow (P.H.: pump head of centrifugal blood pump). When the cells grew to stationary phase (total cell number $3.5–4.1 \times 10^9$), the BAL experiment was carried out. A BAL with one batch of reactor culture was used for a series of four experiments at 1-week intervals. Domestic piglets (approximately 25 kg, 2–3 months old) were used. After the portal flow was by-passed to the jugular vein with a centrifugal blood pump, the portal vein, common hepatic artery, and common bile duct were ligated. Three hours after total liver ischemia, the BAL treatment was started. Blood access was performed using a double lumen catheter inserted into the right atrium via the right jugular vein. Outflow of blood was led to a hemodialysis console at 15 ml/min, and plasma was separated at a rate of 3 ml/min by hollow fiber column. The plasma was passed through the BAL and returned to extracorporeal circulation. Extracorporeal volume (200 ml in the tube and 550 ml in the bioreactor including hollow fiber ventilation unit) was primed with Sublood B. During the first 3 h from the beginning of BAL treatment, the animals were transfused with 500 ml of freeze-stored pig plasma at a rate of 167 ml/h.

trate, and 50 ml/min for infusion. Inorganic solution, Sublood B, was used for dialysis fluid and infusion. The sustentation protocol was the same as that for nontreatment, cell-free BAL, and BAL treatment according to the method described in the previous report (18).

Monitoring Physiological Conditions and Determining Survival Time

Physiological indices such as heart rate, blood pressure, and intracranial pressure were continuously recorded using a bedside monitor (Model66S, Hewlett Packard, Palo Alto, CA, USA). Blood samples were taken just prior to the liver ischemia and at 3-h intervals throughout the experiment. The endpoint of survival for the animal was taken as the time either when systolic pressure fell below 50 mmHg (referred to as "active survival time") or when the heart beat stopped (referred as "gross survival time") as was defined previously (18). The survival hours are expressed as mean and standard deviation.

Analysis of Blood Ammonia and Coagulation Indices

Activated clotting time (ACT) was determined by Hemochron 401® (International Technodyne, Edison, NJ, USA), and activated partial thromboplastin time (APTT) was determined with the assay protocol for human coagulation indices as described elsewhere (1).

Statistical Analysis

Dunnet type multiple comparison with Bonferroni adjustment in the Peto mortality-prevalence test for differences in active survival times (Table 1), the chi-square test for incidence of brain pressure increase (Table 2), and the Mann-Whitney U test for blood coagulation indices (Fig. 3) were used. All tests were two-tailed and performed using PC-SAS (PC/SAS, SAS/BASE, SAS/STAT Software, Version 8.2, SAS Institute Inc., Cary, NC, USA).

RESULTS

Ammonia Removal Activity of GS-HepG2 BAL

GS-HepG2 cells grew to the stationary phase, as judged by glucose consumption rate, 40–60 days after their inoculation into the circulatory flow bioreactor. Glucose consumption increased from 0.47 g/day at day 7 to 1.72 ± 0.12 g/day in the stationary phase ($n = 4$, day 40–60). Although the glucose consumption rates of GS-HepG2 and wild-type HepG2 were similar, ammonia removal activity was detected only in GS-HepG2. The rate was 36 μmol/h from culture medium and hepatic failure plasma, both of which contained 3 mmol/L ammonia. The rate of ammonia decrease by GS-HepG2 was approximately 15% of primary porcine hepatocytes (in preliminary data). In contrast, with wild-type HepG2, ammonia level progressively increased at a rate of 25 μmol/h. The final cell numbers of the two batches of bioreactor culture of GS-HepG2 were 3.5×10^9 and 4.1×10^9. Those of wild-type HepG2 were 3.6×10^9 and 3.8×10^9. The cell density was 1×10^7 cells/cm^3 in the bioreactor and 3.8×10^6 cells/cm^2 on the glass fiber cell matrix. After the BAL treatment began, the blood ammonia levels in the GS-HepG2 group were kept lower than that in the cell-free control, although there was no statistical significance (data not shown).

Survival Times of Pigs

Table 1 shows the overall result of active and gross survival times in this experiment. While there are nontreated and cell-free BAL-treated groups as controls, the proper control to compare the BAL efficacy is cell-free BAL group [8.53 ± 2.52 h (active survival time) and 11.98 ± 5.28 h (gross survival time)]. Plasma pheresis treatment that was clinically performed as hepatic support showed no prolongation of the survival [7.19 ± 3.49 h (active survival time) and 9.47 ± 4.12 h (gross survival time)]. Among the five experimental groups, the survival time was longest in the group treated with BAL

Table 1. Active Survival Time of Pigs With Ischemic Hepatic Failure

Group (n)	Active Survival Time (h)	Gross Survival Time (h)
Nontreated (8)	7.61 ± 2.56*	8.18 ± 2.58§
Cell free (9)	8.53 ± 2.52†	11.98 ± 5.28
PE + CHDF (8)	7.19 ± 3.49‡	9.47 ± 4.12#
W-HepG2 (8)	9.78 ± 4.31	10.54 ± 4.23
GS-HepG2 (8)	14.52 ± 5.28	16.38 ± 6.22

Values are mean ± SD. Data of nontreated group are from our previous report (12). Groups abbreviations: Cell free, treated with cell-free BAL; PE + CHDF, treated with plasma exchange and continuous hemodiafiltration; W-HepG2, treated with BAL of wild-type HepG2; GS-HepG2, treated with BAL of GS-HepG2. Active survival time is defined as the time from completion of hepatic total ischemia to the time when systolic blood pressure was decreased under 50 mmHg. Individual data were: nontreated (4.60, 6.00, 6.00, 6.25, 8.17, 8.20, 8.75, 12.90 h), cell free (4.52, 5.87, 7.10, 7.90, 8.60, 9.32, 10.07, 10.67, 12.75 h), PE + CHDF (4.70, 5.25, 5.53, 5.60, 6.00, 6.20, 9.05, 15.20 h), W-HepG2 (5.53, 6.43, 6.90, 7.42, 8.03, 11.73, 16.03, 16.20 h), GS-HepG2 (4.63, 10.03, 12.83, 15.40, 15.43, 18.23, 19.67, 19.92 h). Gross survival time is defined as the time from completion of hepatic total ischemia to the time of heart beat arrest. Individual data were nontreated (4.65, 6.10, 6.32, 8.40, 8.72, 8.95, 9.20, 13.13 h), cell free (5.00, 9.00, 9.27, 10.08, 10.75, 11.05, 11.28, 20.63, 20.73 h), PE + CHDF (5.18, 6.57, 6.77, 7.17, 7.90, 11.00, 15.37, 15.80 h), W-HepG2 (5.53, 6.43, 8.32, 8.38, 10.70, 11.95, 16.20, 16.83 h), GS-HepG2 (5.83, 12.12, 13.12, 16.23, 16.78, 20.07, 20.63, 26.33 h).
Statistical significance ($p < 0.05$) was determined by Dunnet type multiple comparison with Bonferroni adjustment.
*$p = 0.009$, GS-HepG2 versus nontreatment.
†$p = 0.032$, GS-HepG2 versus cell free.
‡$p = 0.002$, GS-HepG2 versus PE + CHDF.
§$p = 0.009$, GS-HepG2 versus nontreated.
#$p = 0.036$, GS-HepG2 versus PE + CHDF.

containing GS-HepG2 cells [14.52 ± 5.28 h (active survival time) and 16.38 ± 6.22 h (gross survival time)]. The p values on active survival times between this group and the groups with nontreatment, cell-free BAL, and plasma pheresis were 0.009, 0.032, and 0.002. Survival time with GS-HepG2 BAL was 1.5 times longer than with wild-type HepG2 BAL [9.78 ± 4.31 h (active survival time) and 10.54 ± 4.23 h (gross survival time)], although there was no significance between these groups ($p = 0.189$ and 0.162, respectively).

Whereas the gross survival time of GS-HepG2 BAL was the longest, the statistical significance was only detectable toward nontreatment and plasma pheresis groups ($p = 0.007$ and 0.036, respectively). Because the proper control of BAL treatment group is cell-free BAL treatment, the effectiveness of BAL was not confirmed as far as the survival endpoint was taken as the cessation of heart beat.

The difference between active and gross survival times ranged from 0.48 to 9.96 h (3.44 ± 3.25 h, mean ± SD) in the cell-free BAL group, and from 0.29 to 6.41 h in the GS-HepG2 BAL group (1.87 ± 1.92 h), indicating that the terminal stage, with low systolic blood pressure, was short in the GS-HepG2 BAL-treated group.

When survival data were compared between the BAL-treated groups (GS-HepG2 BAL and wild-type HepG2 BAL) and the others (nontreatment, cell-free BAL, and plasma exchange), active survival time was significantly prolonged in the former groups (12.05 ± 5.37 h vs. 7.81 ± 2.91 h, $p = 0.0187$). To detect the unknown but stable effect of cellular biological activities, the cell-free and cell-treated data are compared below.

Incidence of Increased Brain Pressure

There were two types of brain pressure profiles during the progression of hepatic failure: increased type and flat type (Fig. 2). In the increased type, systolic blood pressure dropped markedly just after the peak of brain pressure, followed by a steady low-level state. Half of the cases without cell treatment were the increased type (Table 2). In contrast, only 2 out of 16 cases in the cell treatment group were the increased type ($p = 0.034$ by chi-square test).

Improvement in Blood Coagulation Indices

When blood coagulation indices of surviving animals at 12 h after liver ischemia were compared between the BAL-treated groups and the others, there was significant improvement in APTT and ACT (Fig. 3). However, hepaplastin and factor VII decreased similarly, both in the cell-treated and the other groups (data not shown).

DISCUSSION

We have examined the efficacy of recombinant GS-HepG2 as BAL reactor cells (8,17). While the previous works indicated a sign of improvements in physiological indices, the present report demonstrated statistically significant results on survival time, incidence of increased brain pressure by introduction of the concept of "active survival time," and accumulation of experiments.

As we mentioned in the previous report (18), indeed the survival time of animals is one critical index of BAL efficacy, yet the cessation of heart beat is not a proper endpoint. Because the aim of BAL treatment is to rescue patients, not just prolongation of life, the endpoint should be set before the point of no return. According to our careful observation and detailed analysis, the pigs with systolic pressure over 50 mmHg are able to respond to the bolus transfusion of glucose to increase the pressure (18). Therefore, we embrace the concept of active survival time to estimate the BAL efficacy.

Ammonia is a classical endogenous toxin that may be responsible for the etiology of hepatic coma. Because ammonia removal activity is intrinsic to hepatocytes, this activity is also essential in BAL function. Our original purpose was to remove ammonia from hepatic failure plasma using the BAL with HepG2-supplemented ammonia removal activity. Although hemodialysis can remove ammonia in blood, other low molecular substances are also dialyzed, regardless of their physiological importance. Using the porcine model, we performed the BAL treatment in a closed extracorporeal circuit, obtaining two important results that conventional plasma pheresis does not achieve (i.e., the support of multiple liver functions and the preservation of potentially beneficial substances in blood circulation).

By large-scale culture of GS-HepG2, we obtained ammonia removal activity at 36 μmol/h, which is 1/4 of the total ammonia increase in the pig (140 μmol/h), as calculated from the previous report (19). The mean blood level was kept lower in the GS-HepG2 BAL group than in the wild-type HepG2 BAL group, although there was no statistical significance (8). Nevertheless, active survival time was prolonged significantly in the GS-HepG2-treated group. In addition, when the experimental groups were divided into the cell treatment group and others, the former exhibited significantly increased survival times. Together with the reduction in the incidence of increased brain pressure, the cell-containing BAL improved hepatic failure at the end stage. The reason for this improvement is not clear at present, but may be partly due to a removal of cytokines by the cells in the BAL.

Interestingly, an improvement was also seen in the blood coagulation indices, APTT and ACT (8,17). We hypothesize that the BAL suppressed endogenous consumption of coagulation factors by an unknown mechanism. Although synthetic activity of proteins, such as transferrin and albumin, is low in HepG2 (our preliminary data), certain physiological factors may be respon-

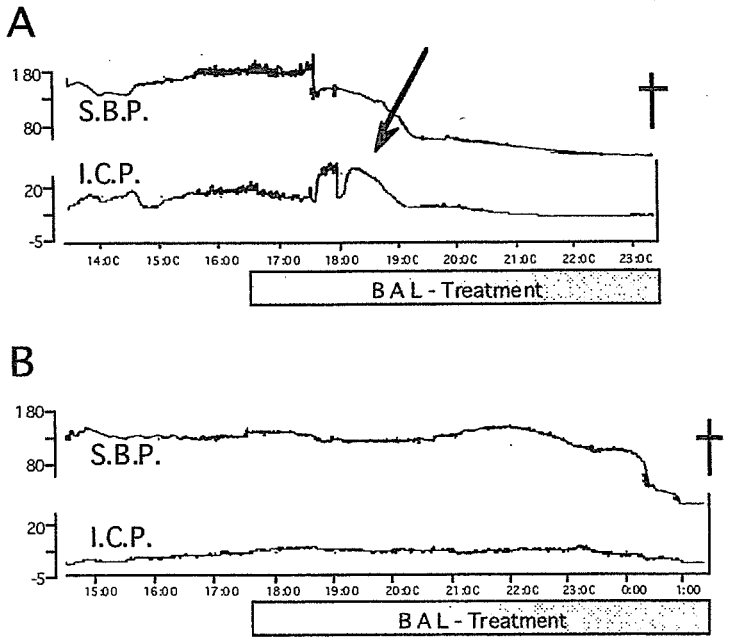

Figure 2. Typical two patterns of brain pressure change. (A) Increased brain pressure (arrow) was observed prior to the rapid decline of systolic blood pressure. (B) Brain pressure was almost unchanged during the experiment. S.B.P.: systolic blood pressure; I.C.P.: intracranial pressure. †Time of cardiac arrest.

sible for prolongation of survival time. Actually, we determined that the culture supernatant of GS-HepG2 induced rat hepatocyte proliferation in vitro and in vivo (11).

The cell numbers required in the BAL to support severe hepatic failure is still unclear in the present experimental model. One enigma in the research of BAL and cell transplantation is discrepancy of cell ability and the efficacy. In this report, too, the calculated efficacy of the BAL is too low to support the whole animal body, but significant prolongation and decreased incidence of brain pressure rise was observed. A minimum requirement for survival was reported to be 10% of total liver weight after extensive partial hepatectomy in rats (13), in which model, however, the remnant hepatocytes were almost intact. For BAL usage, a greater number of hepatocytes may be necessary, because the cell activity is decreased by their isolation and the culture process. In the hepatocyte transplantation model in rats, the effective number of cells ranged from 4×10^6 to 7×10^7, which was estimated to be 0.2–3.5% of the total parenchymal cells in the normal liver [reviewed in (3)]. Moreover, nonhepatic cells, such as splenic lymphocytes and bone marrow cells, were reported to be effective in the treatment of hepatic failure (14). Although the mechanism for this is not completely understood, normalization of the cytokine network may be involved. Further study, including our own, will be necessary to elucidate these phenomena.

Table 2. Decrease of Cases With Rise of Intracranial Pressure

Intracranial Pressure	BAL Treated	Others
Increase	2	11
No increase*	14	14

BAL treated: groups treated with GS-HepG2 and wild-type HepG2 BAL. Others: Nontreated control and groups treated with cell-free BAL and plasma exchange.
*$p = 0.034$ by chi square test.

The lined cells established from either tumor tissue or immortalized normal cells possess an intrinsic risk of virulence by oncogenes. In this experiment, we returned the plasma directly to host circulation after incubating in the BAL. For clinical application, a filtration system or other device to eliminate the risk must be considered. The ideal source of bioreactor cells for BAL would be hepatic stem cells, which have the ability to grow and differentiate. Because most BAL modules are designed to integrate nondividing hepatic parenchymal cells, long-term survival of the reactor cells is not expected.

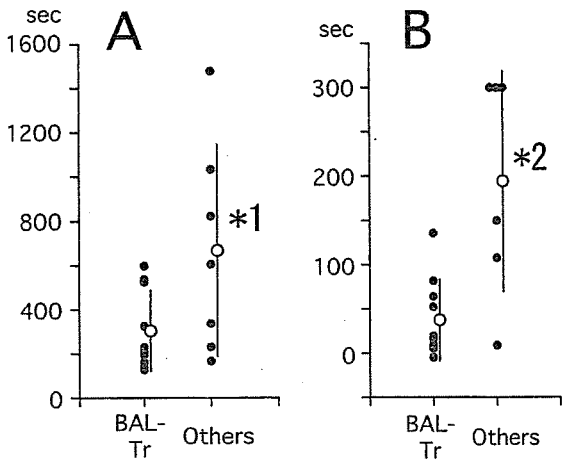

Figure 3. Comparison of blood coagulation indices between BAL treatment and the other groups. (A) Activated clotting time (ACT), *1: $p = 0.040$. (B) Activated partial thromboplastin time (APTT), *2: $p = 0.017$. Closed circles indicate individual data of surviving animals 12 h after hepatic ischemia. Open circle and bar indicate mean ± SD. Statistical significance was determined by Mann-Whitney *U* test.

From this point of view, our circulatory flow bioreactor is suitable for long-term culture of growing cells, including hepatic stem cells.

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Double-Compartment Cell Culture Apparatus: Construction and Biochemical Evaluation for Bioartificial Liver Support

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Functional demands on a bioartificial liver support (BAL) device are not limited to biosynthetic activities, but must also encompass metabolic removal of potentially toxic substances. For most BALs, however, the concept and design are exclusively directed to biosynthetic support. To add the ability to metabolize and remove toxic substances, we designed a double-compartment cell culture apparatus (DCCA). Two compartments are separated from each other by a compact epithelial cell sheet spread over a synthetic microporous membrane. When a renal proximal convoluted tubular cell line that had been transduced with the human multidrug-resistant (MDR) gene, PCTL-MDR, was introduced into one of the compartments (hereafter referred to as the "inner" compartment) of the DCCA, a compact cellular monolayer was formed on the membrane. Ammonium ions passed across the membrane, but glucose and its metabolite lactate could not, indicating that the DCCA allowed selective transportation of cellular metabolites. In addition to PCTL-MDR, HepG2, a cell line of hepatic-origin, transduced with CYP3A4 (designated GS-3A4-HepG2), was seeded on the opposite side of the membrane, and the metabolism and transportation of lidocaine were studied. The lidocaine metabolite, monoethylglycinexylidide, was detected in the inner compartment across the PCTL-MDR cell layered membrane, indicating that metabolism and the selective transportation of metabolites between the two compartments occurred by cooperation of renal and hepatic cells. These results suggest that this type of DCCA represents a novel BAL that possesses biotransporting activities, as well as biosynthetic and metabolic activities.

Key words: Bioartificial liver support; Double-compartment cell culture apparatus; Toxic substance removal; Biotransport activity

INTRODUCTION

Progress in modern biotechnology and medicine enables artificial devices to support patients at the end stage of organ failure (12,17). One major application of these devices is the support of liver function. There are two concepts in extracorporeal artificial liver support: the nonbiological approach or the cell-based hybrid approach. The former has been developed as plasmapheresis (9) and the latter as bioartificial liver support (BAL) (2,16). Over the past few years, some cell-based BALs succeeded in providing life support for patients with fulminant hepatic failure waiting for liver transplantation (2,4,13,16). Moreover, temporary support with a BAL may allow time for the patient's own liver to regenerate without need for transplantation.

A typical type of BAL is composed of a hollow fiber module in which hepatocytes are packed either outside or inside the hollow fiber tubules, and the patient's plasma or blood interacts with the cells through the hollow fiber membrane (4,16). In another model, hepatocytes are cultured on support matrices such as small beads or membranes, and patient plasma is applied directly (2,11,15). Previously, we developed a BAL of fixed bed perfusion reactor type, which essentially belongs to the latter model, and succeeded in extending the survival time of pigs with ischemic liver failure (7).

The above two BAL models carry out hepatic biosynthesis and metabolism, but cannot actively transport toxic substances. During the development of our BAL, we noticed that hepatocytes alone are not sufficient for achieving biological transportation. Therefore, in addition to

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hepatocytes, we chose a cell line of kidney proximal convoluted tubule origin, PCTL-MDR, to perform metabolism and active transportation tests. The cell line, PTCL, was first established from rabbit kidney proximal cells by transduction of SV40T antigen, and further transduced with multidrug-resistant (MDR) gene to increase anion transport activity, and therefore termed PCTL-MDR (19). In a small-scale experiment, the monolayer membrane of PTCL cells successfully transported a model toxic substrate, digoxin, and hydroxytestosterone, which was produced by recombinant HepG2 (5,6). To develop a preclinical scale module that is applicable for experimental animals such as rabbits and pigs, we designed and constructed a double-compartment cell culture apparatus (DCCA) with a membrane separating the two compartments on which actively transporting cells are layered. This communication describes the construction and functional characteristics of such a DCCA and discusses prospects for use of this novel module design.

MATERIALS AND METHODS

Brief Description of the Double-Compartment Cell Culture Apparatus (DCCA) and Circulation System

The body of the DCCA consists of a pair of 5-mm-thick glass plates (150 × 250 mm) and a pair of size-matched 3-mm-thick silicon molds with a square window (110 × 210 mm) as shown in Figure 1. Expanded polytetrafluoro-ethylene (ePTFE) microporous membrane (Fluoropore, FP100, Sumitomo Electric Industry, Osaka, Japan) was set between the two silicon molds so that it divided the inner space into two compartments in the middle. The average pore size of the ePTFE membrane was 1 μm and thickness of 75 μm. There was a conformable zigzag flow pass (32.5 mm width) in each compartment. The volume and area of flow pass were 64.5 cm^3 and 215 cm^2, respectively. These components (i.e., glass plates, silicon molds, and an ePTFE membrane) were held tightly together in aluminum frames with clamps to form the DCCA. For autoclaving, they were put together loosely, and tightened after cooling.

The DCCA possesses a pair of outlets and inlets for each compartment (Fig. 2). Medium is circulated independently through each compartment by way of separate reservoir bottles (500 ml each), using a peristaltic pump (Master Flex L/S 7520-10) with a 7012-20 type pump head (Cole-Parmer Ins., IL, USA). Reservoir bottles were supplied with 5% CO_2/95% O_2 gas continuously to adjust medium pH to 7.2. Each circulation line has a cell inoculation port and two air-trap chambers. The entire system, including reservoir bottles, can be placed in an incubator at 37°C. DCCA were placed vertically to avoid accumulation of air bubbles, except at the time of cell inoculation.

Flow Control and Membrane Permeability Test

After DCCA assembly, a solution of 0.15 mol/L NaCl was constantly circulated at the rate of 10 ml/min. To visualize medium flow, 1 ml of blue dextran (10 mg/ml, Sigma) was added directly to the circulation medium through the inlet tube.

To determine the diffusion rate of small molecules across the cell-free ePTFE membrane in the DCCA, a test solution (0.15 mol/L NaCl containing either 0.2 mol/L calcium lactate, 1.0 mmol/L ammonium sulfate, or 17.5 mmol/L glucose) was circulated in one of the two circuits at the rate of 10 ml/min. NaCl solution flow in the other compartment was at the same rate and samples were collected from each reservoir at 4-h intervals. Time-dependent change of lactate concentration was determined using Lactate Pro Test Strip (ARKRAY Inc, Kyoto, Japan), that of ammonium ion concentration by using an AMICHECK Meter (ARKRAY Inc), and of glucose concentration using the anthrone-sulfuric acid method (3). Diffusion rates for lactate, ammonium, and glucose were calculated from these values.

Cell Culture Experiments

Before inoculation into the apparatus, cells were grown in 175-cm^2 culture flasks with a mixture (1:1) of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM/F12 medium, Invitrogen, Carlsbad, CA, USA), supplemented with 5% fetal bovine serum (Invitrogen). GS-3A4-HepG2 and PCTL-MDR cells were cultured in the presence of 200 μg/ml Zeocin™ (R250-01, Invitrogen), or 40 μg/ml neomycin (Sigma, St. Louis, MO, USA), respectively. Cells at the subconfluent stage were harvested with 0.25% trypsin (15090-046, Invitrogen) for inoculation.

The sterilized DCCA was first filled with 95% ethanol to remove micro air bubbles in the ePTFE membrane. It was washed with phosphate-buffered saline and then filled with DMEM/F12 culture medium containing 5% fetal bovine serum.

Two different cell lines were inoculated successively on the opposite sides of the ePTFE membrane in a DCCA placed horizontally. First, 1.0×10^8 kidney-derived PCTL-MDR cells in 100 ml were introduced into one side of the compartment through the circulation system using a peristaltic pump. After the inoculation, the apparatus was gently shaken for 5 min to allow the cells to spread evenly over the membrane. The apparatus was kept static for 1 h and medium circulation started at a flow rate of 1 ml/min for 3 h. The circulation rate was raised to 5 ml/min for an additional 21 h. Next, the DCCA was turned upside down, and 1.0×10^8 GS-3A4-HepG2 cells were inoculated into the other compartment using the same procedure as for PCTL-MDR. Circula-

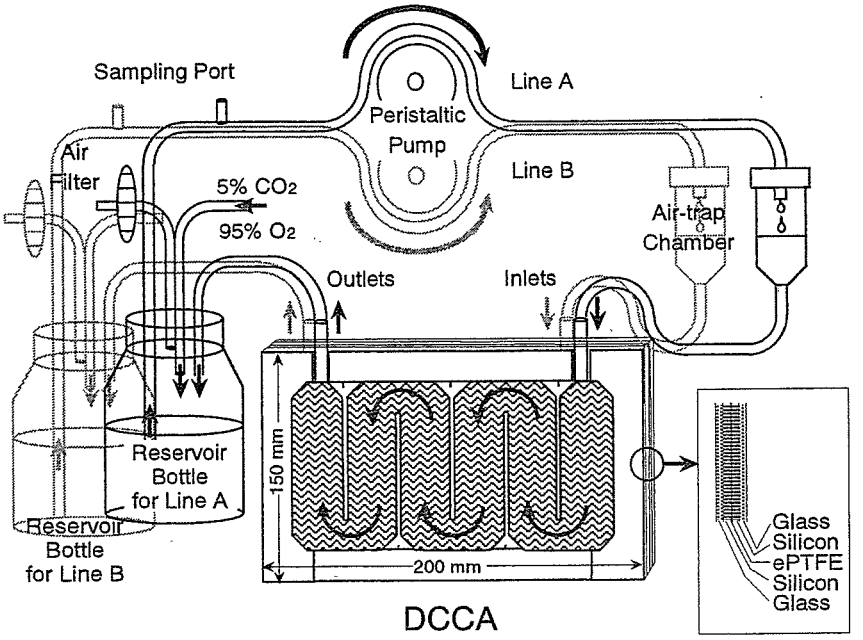

Figure 1. Schematic illustration of double-compartment cell culture apparatus (DCCA) and circulation system. A pair of symmetric culture chambers with zigzag flow path is formed on each side by piling up a pair of silicon molds and glass plates as shown in cross section (inset). The chambers were divided by an expanded polytetrafluoro-ethylene (ePTFE) membrane in the middle. Each chamber is supplied with medium through an independent line, Line A and B. The volume and area of flow pass were 64.5 cm^3 and 215 cm^2, respectively.

tion of medium on the PCTL-MDR side was stopped during the second inoculation. Twenty-four hours thereafter, the apparatus was placed vertically and medium flow started on both sides (5 ml/min). Hereafter, the ePTFE membrane side on which PCTL-MDR cells were attached is referred to as the outer side, and the other side on which GS-3A4-HepG2 cells were attached as the inner side. Concomitantly, the compartment and the circulation of the outer side is referred to as the outer compartment and outer circuit, and those of the inner side as the inner compartment and inner circuit, respectively. According to preliminary experiments, 1.0×10^8 cells of PTCL-MDR reached confluence after 3 days of incubation.

During the culture period, unless otherwise stated, a medium reservoir bottle was changed every other day and a new bottle with 500 ml fresh medium substituted. Samples (5 ml) were taken from the two reservoir bottles at the times indicated, and stored frozen at –20°C until HPLC analysis. In some experiments, lidocaine (final concentraton, 100 μmol/L) (Alexis Biochemicals, Montreal, Canada) was added as a test substance to in-

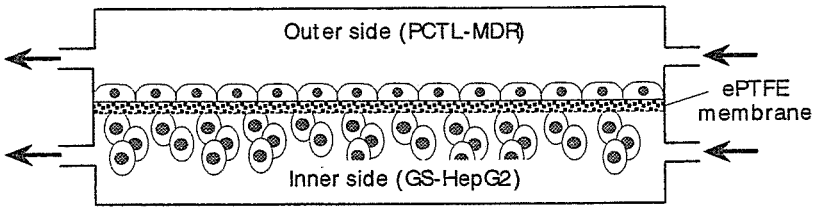

Figure 2. Cross section of DCCA. The inside of the vessel is divided by the ePTFE membrane and PCTL-MDR and GS-HepG2 cells are cultured on opposite sides. The PCTL-MDR side and GS-HepG2 side are termed outer and inner side, respectively.

vestigate its metabolism and transportation across the membrane.

HPLC Analysis

Lidocaine and its metabolites in the culture medium were analyzed by a C18 reverse-phase column (particle size 5 μm, internal diameter 4.6 × 150 mm) (Inertsil ODS-3V, GL Sciences, Tokyo, Japan), using an HPLC system (Model 805, Waters, MA, USA). Prior to HPLC analysis, 2 ml of the medium was partially purified with a Sep-Pak Plus (Waters Corp., Milford, MA, USA), and 25 μl of the sample thus obtained applied to the column. Samples were eluted with 15% acetonitrile in 20 mmol/L $NaClO_4$ solution (pH 2.5) at a flow rate of 2.0 ml/min at 40°C, and lidocaine and its metabolites detected at a wavelength of 205 nm (Type L-4000 UV detector, Hitachi, Tokyo, Japan). The authentic standard of lidocaine major metabolite, monoethylglycinexylidide (MEGX), was a generous gift from Astra Pharmaceuticals (Wayne, PA, USA).

RESULTS

Examination of Medium Flow

After the initial DCCA conception and design, several prototypes were constructed and tested. Improvements were mainly carried out on the shape of flow pass to remove air bubbles and to smooth medium flow. To remove air bubbles, inlet and outlet ports were changed from single tube form to branched tube form. To obtain a smoother flow, the shape of the corner of the compartment was changed from rectangular to trapezoidal (Fig. 3A). Although medium still flowed slightly faster in the middle of the stream, no dead space was now seen at any place in the compartment (Fig. 3B).

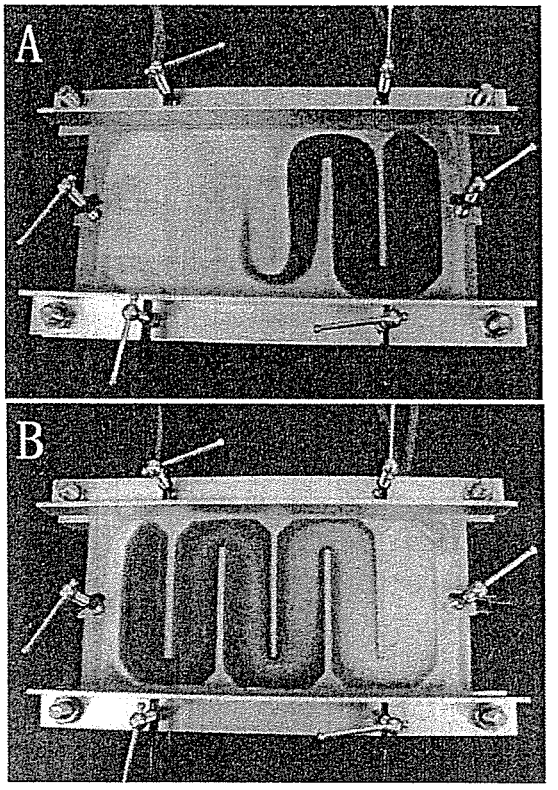

Figure 3. Examination of medium flow by blue dextran solution. One milliliter of blue dextran (10 mg/ml, Sigma) was added directly to a constant flow of 0.15 mol/L NaCl at a rate of 10 ml/min through an inlet tube. The time interval between (A) and (B) was 1 min.

Passive Diffusion Across Cell-Free Membranes

As shown in Figure 4, the concentration of lactate achieved equilibrium between the two compartments by 28 h and ammonium and glucose by 20 h. Thus, the passive diffusion rate, P, was calculated from the time needed for the difference in metabolite concentration between the two compartments to be reduced by half, $t_{1/2}$; the surface area of the membrane, S; and the volume of reservoir, V; using the following equation: $P = \ln(1/2) \times V/S \times t_{1/2}$. It was calculated to be 5.1×10^{-4}, 5.0×10^{-5}, and 8.5×10^{-5} cm/s, respectively, for lactate, ammonia, and glucose.

Cell-Mediated Metabolism and Transport

When 1.0×10^8 PCTL-MDR cells were seeded on the outer side of the ePTFE membrane, a tight monolayer was formed in 3 days. Fresh growth medium was replenished in the circulation units, and the concentrations of lactate ions, glucose, and ammonium ions were determined every 12 h for the subsequent 7 days of constant circulation. The initial concentrations of lactate ions in both compartments were 0.8 mmol/L (Fig. 5A). After medium circulation began, the concentration level in the inner circuit (HepG2 site) was maintained around 0.8 mmol/L throughout the circulation period. On the other hand, the concentration in the outer circuit (PCTL site) increased steadily and reached 11.0 mmol/L at the end of the 7 days.

The ammonium concentration in the outer circulation was virtually unchanged and the level was maintained at between 4 and 5 mg/dl (mean ± SD, 4.35 ± 0.22 mg/dl) (Fig. 5B). In contrast, the level in the inner circulation medium decreased steadily from 4.6 mgN/dl to 2.7 mgN/dl during the 7 days.

The concentration of glucose in the outer circuit decreased steadily during the 7 days of circulation from 3.0 mg/L to 2.0 mg/L, while it was maintained in the inner circuit at approximately 3.2 mg/L (Fig. 5C).

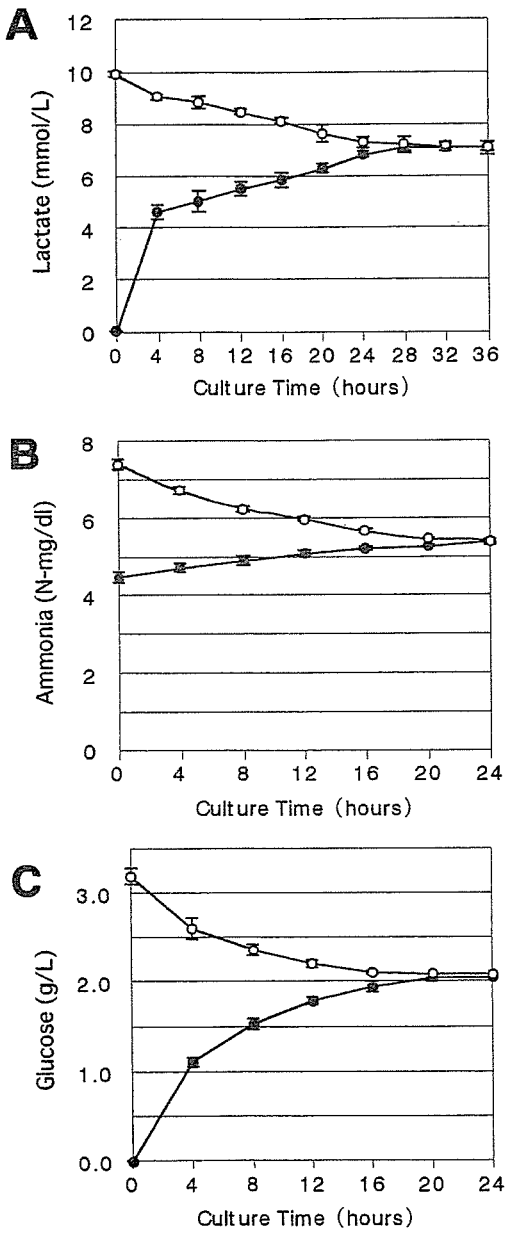

Figure 4. Time course profiles for passive cross-membrane transport of calcium ions (A), ammonium ions (B), and glucose (C). To one of the compartments of the DCCA (open circles), 0.15 mol/L NaCl solution supplemented with 0.2 mol/L lactate calcium (A), growth medium supplemented with 1 mmol/L $(NH_4)_2SO_4$ (B), or PBS supplemented with 17.5 mmol/L glucose (C) was circulated, while to the other compartment (filled circles), only 0.15 mol/L NaCl (A), growth medium (B), or PBS (C) were circulated, respectively. A sample (5 ml) was removed from a reservoir bottle containing 500 ml solution every 4 h for chemical determination. In all experiments, perfusion rate was 10 ml/min. In this experiment, no cells were inoculated.

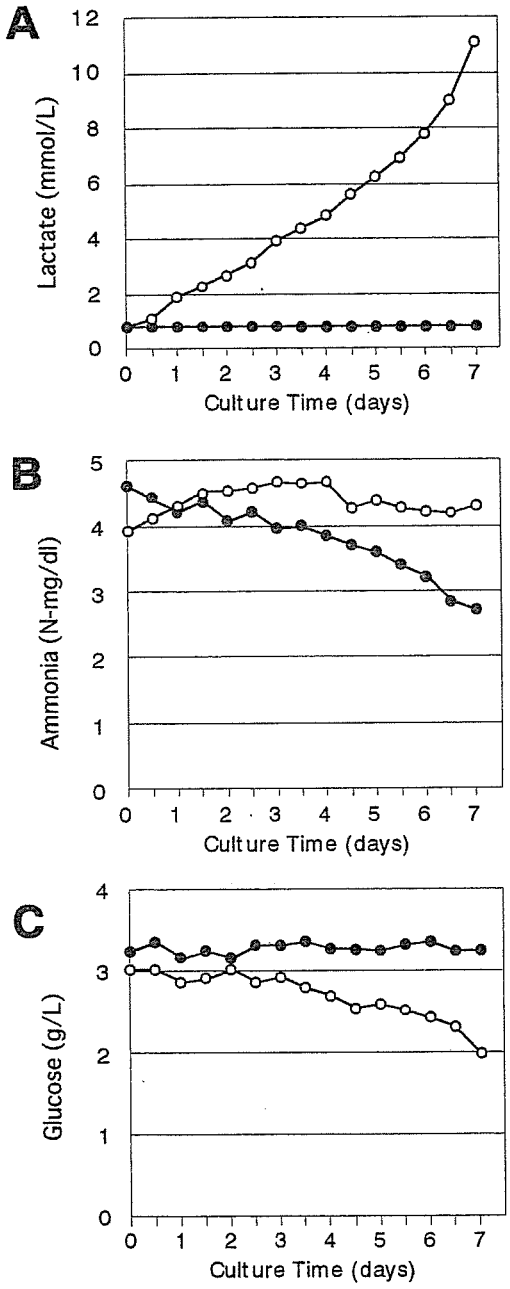

Figure 5. Time course profiles of accumulation and/or consumption of lactate ions (A), ammonium ions (B), and glucose (C) in the outer compartment (open circles) and in the inner compartment (filled circles) of a DCCA seeded with a monolayer and incubated with PCTL-MDR cells on the outer compartment side of its separation membrane.

Lidocaine Detoxification and Transportation

Before studying metabolism and transmembrane transport of lidocaine in the DCCA, a dish-scale experiment was performed. Nine 10-cm dishes, each containing 20 ml growth medium, seeded with GS-3A4-HepG2 cells, and with lidocaine added at a final concentration of 100 μmol/L, were incubated and harvested at 12-h intervals over a total period of 96 h. As shown in an HPLC profile of a sample obtained during the incubation (Fig. 6), lidocaine and its major metabolite MEGX were clearly separated from each other.

The identity of the latter peak with MEGX was confirmed using an authentic sample of MEGX. Approximately one third of the lidocaine added was metabolized during the 96-h period, and approximately 70% of the metabolites recovered was MEGX.

To study the metabolism and transmembrane transport of lidocaine in the DCCA, a suspension containing 1.0×10^8 PCTL-MDR cells was inoculated into the outer compartment of the apparatus, which was then incubated for 24 h to allow formation of a cell sheet on the ePTFE membrane. Next, the same number of GS-3A4-HepG2 cells was inoculated into the inner compartment as described in Materials and Methods. After further incubation for 6 days, lidocaine was added into the inner reservoir bottle, at a final concentration of 100 μmol/L. The whole cell culture system was further incubated for 24 h. Thereafter, the deethylated metabolite of lidocaine, MEGX, as well as lidocaine itself, was detected in both compartments, although only approximately 1% of lidocaine was metabolized to MEGX in this experiment, as shown in Table 1.

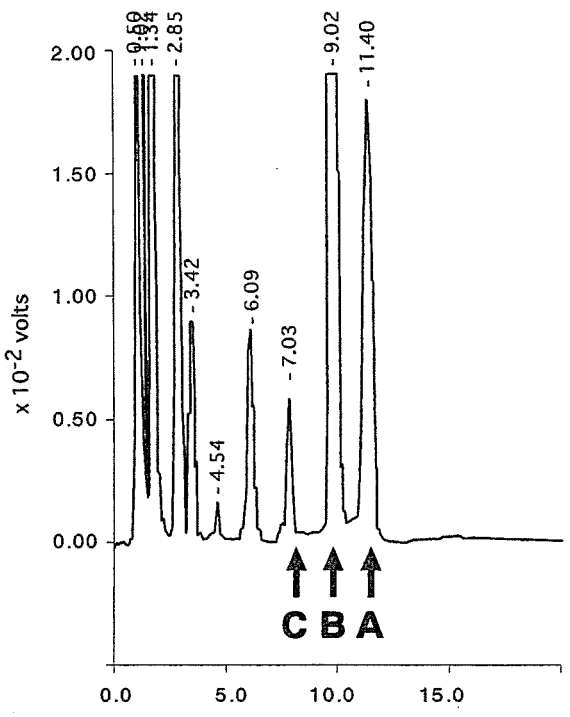

Figure 6. Chromatographic separation of lidocaine and MEGX in culture medium incubated with GS-3A4-HepG2 cells in a culture dish. A 10-cm dish seeded with 7×10^6 of GS-3A4-HepG2 cells was incubated in 20 ml growth medium in the presence of 100 μmol/L (27 μg/ml) lidocaine for 36 h. Peak A is lidocaine, B is phenol red, and C is MEGX.

DISCUSSION

The DCCA described here has several advantageous features as an experimental culture model; for instance, simple assembly, ease of changing the membrane and other materials, and feasibility of inoculating two different types of cells on the opposite sides of the membrane. Previously, using a small-scale static culture, we reported that the coculture of hepatic and renal cells on an ePTFE membrane facilitated the metabolism and one-way transport of testosterone. In the small-scale system, 4×10^6 cells were inoculated on either side of the 3.14-cm^2 membrane (5,6). Here the DCCA was able to maintain approximately 25 times more cells constantly circulating in the culture medium than in the previous static culture unit. We believe that this device may be suitable for preclinical animal experimentation.

The ePTFE membrane has been used for industrial purification of water and for artificial blood vessels in the medical field. In spite of its hydrophobicity, the membrane was suitable as a cell culture substrate (18). The merits of ePTFE membrane as a culture substrate include heat stability, chemical inertness, and mechanical strength. Thus, we believe that ePTFE is one of the best choices as a culture substrate for the DCCA, among all candidate materials, such as polysulfone, polyamide,

Table 1. Formation of MEGX From Lidocaine and its Distribution Among the Two Compartments in the DCCA

	Inner Compartment		Outer Compartment	
	0 h	24 h	0 h	24 h
Lidocaine	99	51	0	36
MEGX (nmol/ml)	0	0.49	0	0.32

There were 1×10^8 3A4-GS-HepG2 cells seeded to the inner compartment and 1×10^8 PCTL-MDR cells to the outer side compartment to form a compact cell layer on the separation membrane. After 48 h of preliminary incubation without the addition of lidocaine, lidocaine was added to the circulation medium of the inner compartment (final concentration 100 μmol/L), and then the system was incubated for 24 h before sampling for HPLC.

polyacrylonitrile (PAN), or polymethyl methacrylate (PMMA) membranes.

Renal proximal cells are known to form a tight monolayer barrier on a synthetic membrane (5,6,8,10,19). Our previous work showed that the monolayer of PCTL-MDR selectively passed digoxin, a typical substrate of anion transporter, MDR1, but did not pass inulin (5). In the present study, we confirmed selective transport by determining the concentration of glucose, lactate ions, and ammonium ions in the DCCA. Evidently in the outer compartment where PCTL-MDR cells were cultured, glucose concentrations were decreased, but lactate increased while in the inner compartment; the levels of these two substances were unchanged. This suggests that the cell layer efficiently prevented cross-membrane passive transportation of these small molecules (Fig. 5A, C). The concentration of ammonium ions decreased in the inner compartment, while the level was unchanged in the outer compartment (Fig. 5B). Physiologically, ammonium ion excretion is known to vary with changes in acid base balance, and to represent the major regulatable component of net acid excretion (20). Thus, we assumed that the concentration gradient of lactate induced the counterflow of ammonium ion, so that ionic balance was maintained.

To add metabolizing capacity to the DCCA, we inoculated GS-3A4-HepG2 cells of hepatic origin, which had been transduced with the CYP3A4 gene (14). Lidocaine is one of the most common indicators of detoxifying activity of liver and is metabolized to MEGX by CYP3A4 (1). As shown in Figure 6, MEGX was detected not only on the GS-3A4-HepG2 side but also on the PCTL-MDR side. At present we do not know whether PCTL-MDR can actively transport MEGX. Nevertheless, the lidocaine addition test unequivocally demonstrated metabolic activity in the DCCA.

In static cultures, 8×10^5 cells/ml GS-3A4-HepG2 cells metabolized 100 μmol/L lidocaine at a rate of 5.83 fmol/h/cell, so by extrapolation, the total activity was 4.66 nmol/h/ml (Omasa, unpublished data). On the other hand, in the present results (Table 1), MEGX concentration increased to 0.49 nmol/ml and 0.32 nmol/ml in the inner and outer compartments, respectively, after 24-h culture. Although 1×10^8 GS-3A4-HepG2 cells were cultured in the DCCA, because of the volume of medium (550 ml), the cell density in the system overall was only 1.8×10^5/ml. In addition to this low cell density, the difference in the medium flow rate and oxygen concentration might affect the metabolic activity of the cells. Thus, we regard the low detoxification rate in comparison with that of the previous small-scale experiment as explainable.

In a preliminary experiment, bilirubin was added to DCCA because it is a putative target substance of BAL. However, unbound bilirubin was highly toxic to the cells in our model, even at a concentration within its physiological range. To render cells resistant to bilirubin, transduction of UDP-glucuronosyl transferase should be effective. We have started to develop HepG2 transduced with this transferase, and to construct a DCCA that will possess bilirubin removal activity.

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