

Selective and Effective Cytotoxicity of Folic Acid- conjugated Cholesteryl Pullulan Hydrogel Nanoparticles Complexed with Doxorubicin in *In Vitro* and *In Vivo* Studies

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ABSTRACT: We previously reported that cholesteryl pullulan (CHP) derivatives were effective carriers drug delivery systems in targeting cancer cells. We have now synthesized folic acid-conjugated CHP hydrogel nanoparticles (FA-CHP). FA-CHP complexed with the anticancer drug doxorubicin (DOX) show a higher cytotoxicity than CHP complexed with DOX in *in vitro* studies. The expression of a folate receptor (FR) is elevated in many cancers; in this case, confocal image analysis revealed that FA-CHP complexed with DOX exhibited greater cellular uptake than CHP complexed with DOX in human epidermal carcinoma (KB) cells over-expressing surface FR. *In vivo* studies showed that the increase of tumor volume in a nude mice xenograft model was significantly

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suppressed. Accordingly, FA-CHP may be an effective vehicle for the delivery of anticancer drugs and has a potential application in the treatment of over-expressing FR solid tumor cells.

KEY WORDS: folate receptor, drug targeting, cholesteryl pullulan, doxorubicin

INTRODUCTION

Recently, targeted drug delivery systems containing liposomes, polymer conjugates or other vehicles have shown great promise in expanding cancer treatment possibilities [1,2]. The use of targeted drug delivery systems leads to a reduction in the effective dose of a drug and any accompanying drug toxicity. However, more effective targeted systems are needed to enhance the cellular uptake of the drug-containing nano-carriers through the various specific sites on the membranes of cancer cells.

Folic acid is an essential vitamin required for transfer reactions in metabolic pathways. The vitamin is consumed in proliferating cells for the biosynthesis of nucleotide bases. It is also well known that FR is over-expressed in various human cancers, including malignancies of the ovary [3], uterus [4] and mesothelium [5]. The degree of FR over-expression is positively correlated with a higher histological grade and more advanced stages of cancer [3].

FR is a 38–40 kDa glycosyl-phosphatidylinositol-anchored glycoprotein with a high affinity for the folic acid vitamin [6]. The family of human FR consists of three isoforms, FR- α , FR- β and FR- $\gamma\gamma'$. FR- α in particular is over-expressed in many cancers [4,7,8]. Therefore, this fact has been exploited to improve drug delivery systems using various carriers such as folate-PEG and folate-liposomes [9,10]. Sunamoto et al. developed hydrophobic polysaccharides as an effective drug delivery system [11,12] and have reported that CHP derivatives can be applied for selective drug delivery in rat and human hepatoma cell lines (AH66: rat hepatoma; HuH7: human hepatoma) in both *in vitro* and *in vivo* studies [2,13,14].

Subsequently, we synthesized a FA-CHP [15]. The purpose of this study was to clarify whether FA-CHP complexed with an anticancer drug exhibits selective cytotoxicity via FR against cancer cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell Culture

KB cells (TKG 0401) from a human epidermoid carcinoma that overexpresses FR [7] were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, in Sendai, Japan. For *in vitro* studies KB cells were cultured in folate-depleted Dulbecco's modified Eagle's medium (FD-DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO, Invitrogen Co., New York), penicillin G (100 units/L), streptomycin (100 μ g/L), amphotericin B (125 μ g/L) (GIBCO, Invitrogen Co., New York) and 1.5 mM HEPES in a humidified atmosphere containing 5% CO₂ at 37°C. FD-DMEM was prepared in our laboratory according to the composition of Nissui Pharmaceutical Co., Ltd, Tokyo except for 4 μ g/L addition of folic acid instead of 4 mg/L. For maintenance, KB cells were cultured in DMEM with 10% FBS, penicillin G, streptomycin and fluconazole.

Synthesis of FA-CHP

FA-CHP used in this study was prepared as reported previously [15] with slight modifications. Briefly, CHP (8 g) was activated by 4-nitrophenyl chloroformate (PNPC), before conjugating with folic acid γ -2-aminorthylamide (EDA-FA). The activated CHP was dissolved in dimethylsulfoxide (DMSO)/pyridine (1:1) and EDA-FA was added into the solution, followed by the addition of 4,4-dimethylaminopyridine (DMAP). The solution was stirred for 4 days in the dark at room temperature. After quenching the reaction with ethanol and standing the mixture overnight, the yellowish precipitate was collected by centrifugation, washed twice by ethanol and dried. The precipitate was dissolved in 0.01 M NaOH (pH was adjusted to approximately 11.5) and stirred for 12 h, the solution was then dialyzed against 0.01 M NaOH for 4 days. The dialyzed solution of FA-CHP was neutralized and purified by a Sephacryl S-500HR column. The collected fractions of FA-CHP were combined and concentrated by evaporation. The concentrated solution of FA-CHP was then dialyzed against distilled water for 3 days, and dried (yield 1.8 g; the folic acid substitution ratio was 0.4% per glucoside unit).

Preparation of FA-CHP, CHP, DOX Complexes

DOX was a gift from the Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. Cholesterol-bearing pullulan (CHP-108-0.9, Lot000125) was a gift from the NOF Corporation, Tokyo, Japan. For *in vitro* studies, the procedure for the preparation of FA-CHP/CHP/DOX complexes was as follows. First, FA-CHP (1 mg) and CHP (4 mg) were added in 0.2 mL of DOX solution (0.1 mg/mL) in which the phosphate buffered saline (PBS) solution was used and the mixture was capped and incubated at room temperature overnight. The CHP and FA-CHP coaggregated to internalize DOX into nanoparticles 20–30 nm in size [11]. Then 0.8 mL PBS was added to the mixture, which was further incubated at 37°C with gentle shaking. This formulation of the mixture of FA-CHP/CHP/DOX complexes (weight ratio: 1.00:4.00:0.02) was used directly after filtration to remove the unencapsulated DOX [2]. For *in vivo* studies, the preparation of CHP/DOX complexes and FA-CHP/DOX complexes was performed in the same way.

MTT Assay for *In Vitro* Studies

The MTT assay is a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt (MTT) to blue formazan crystals. KB cells were seeded into 96-well plates, and at various times after the drug delivery agents were added, a 0.4% MTT dye and 0.1 M sodium succinate (1:1) solution was also added. The plates were incubated for 3 h in a humidified chamber at 37°C, after which time the medium in the plates was aspirated and the dye eluted with DMSO and 2 M KOH (1:1). Absorbance was measured at 450 nm (Bio-RAD).

Cytotoxicity of FA-CHP and CHP

KB cells were seeded into 96-well plates at a cell density of 2×10^4 /mL in FD-DMEM. After 24 h the medium was changed and the agent (CHP 5.00 mg/mL or FA-CHP 5.00 mg/mL) was added to 1% of the medium (200 μ l). The MTT assay was performed 1, 3 and 5 days after drug administration.

In Vitro Effects of FA-CHP/CHP/DOX Complexes

KB cells were seeded into 96-well plates at a cell density 2×10^4 /mL in FD-DMEM. After 24 h, the medium was changed and 2 μ L of one of the following additives was added to 1% of the medium (200 μ L): PBS,

DOX (0.02 mg/mL), CHP/DOX complexes (weight ratio: 5.00:0.02 mg/mL) or FA-CHP/CHP/DOX complexes (weight ratio: 1.00:4.00:0.02 mg/mL). The MTT assay was performed 1, 3 and 5 days after the addition of the agents.

Cellular Uptake of FA-CHP/DOX Complexes

To investigate the cellular uptake of DOX, cancer cells were examined under a confocal microscope (Carl Zeiss LSM510, Germany). KB cells were seeded in a glass-bottomed dish at a cell density of 1×10^5 /mL and incubated for 24 h. After pre-incubation, the medium was removed and the drug suspension was added in a folate-depleted medium and in a folate-overdosed medium. The cells were incubated for 1 h at 37°C and then washed twice with cold PBS. To visualize cellular uptake of DOX, the cancer cells were examined by confocal microscopy with an excitation wavelength of 488 nm and an emitting wavelength at 560–615 nm.

Therapeutic Efficacy of FA-CHP/DOX Complexes in a Xenograft Model

We used female athymic BALB/c mice (nu/nu, body weight = 20–25 g) in these studies. The mice were purchased from Charles River Laboratories and maintained on a folate-free rodent diet, which is based on AIN-26 without folic acid, on arrival and in all runs. To implant tumor cells, 1×10^6 KB cells were injected subcutaneously in the right femoral region of the mice using a 26-gauge needle. All experiments were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee. All animals were maintained in a pathogen-free environment at the Laboratory Animal Center for Frontier Life Sciences, Nagasaki University. Mice (in groups of five) received the following treatment when the tumors reached palpable sizes of 10–50 mm³ after implantation: (a) PBS (200 mg/kg), (b) free DOX (2 mg/kg in DOX), (c) CHP/DOX complexes (CHP 200 mg/kg, DOX 2 mg/kg), and (d) FA-CHP/DOX complexes (FA-CHP 200 mg/kg, DOX 2 mg/kg). The drugs were given by intravenous injection on every fourth day for a total of six doses (days 1, 5, 9, 13, 17 and 21). Tumor size was measured on every fourth day, and calculated using the following equation: $\text{volume} = 0.5 \times d1 \times d2^2$ ($d1$ is the longest length, $d2$ is the shortest length). Mice were sacrificed on the 29th day after the initial treatment and the tumor weights

were measured. Statistical analyses were performed by the Mann-Whitney U-test with Bonferroni correction after multivariate analysis with the Kruskal-Wallis H-test. Statistical significance was set at the $p < 0.05$ level.

RESULTS AND DISCUSSION

The FA-CHP used in this study was prepared as reported previously [15], with slight modifications. The chemical structure of FA-CHP is shown in Figure 1. The carriers (CHP, FA-CHP) alone did not suppress the growth of KB cells, as shown in Figure 2. Furthermore, no cytotoxic effects were evident in the cancer cell lines used.

Selective Cytotoxicity of FA-CHP/CHP/DOX toward KB Cells

The formulation of a mixture of FA-CHP/CHP/DOX (weight ratio: 1.00:4.00:0.02mg/mL) significantly inhibited the growth of KB cells compared to the mixture of CHP/DOX (weight ratio: 5.00:0.02mg/mL) and to free DOX (0.02mg/mL) at day 5 in folate-depleted DMEM (as shown in Figure 3).

There were no differences in the cellular uptake of DOX when KB cells were incubated with free DOX or CHP/DOX in a folate-depleted medium (Figure 4). For FA-CHP/DOX, fluorescent labeled KB cells were more clearly visualized in a folate-depleted medium than in a

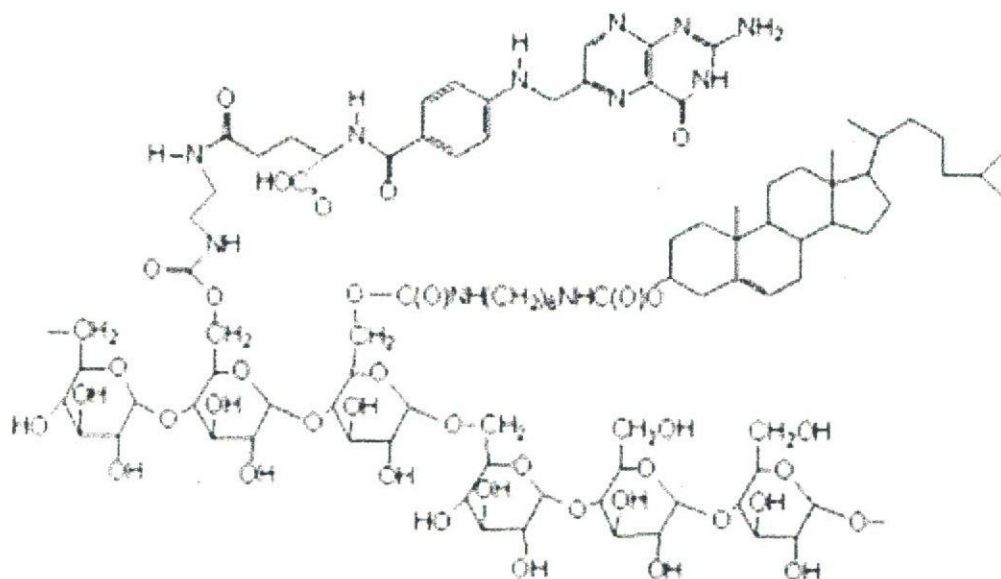


Figure 1. Chemical structure of FA-CHP.

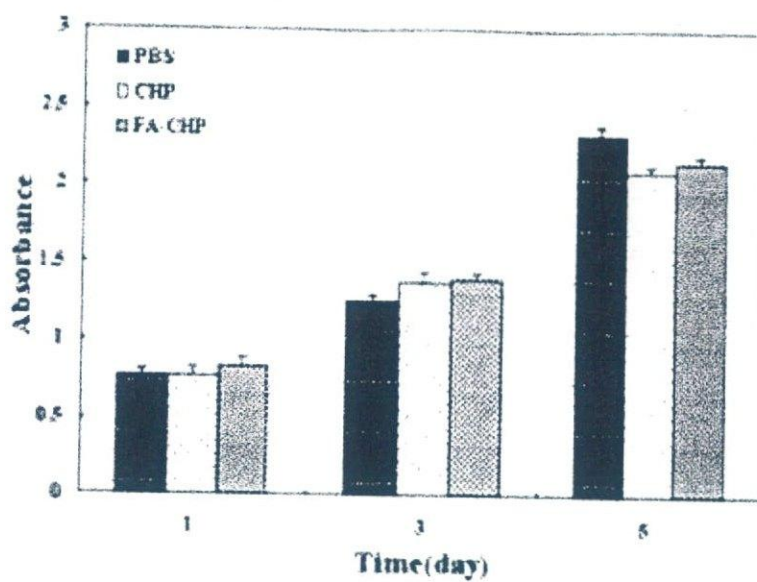


Figure 2. Cytotoxicity of CHP and FA-CHP toward KB cells in a folate-depleted medium.

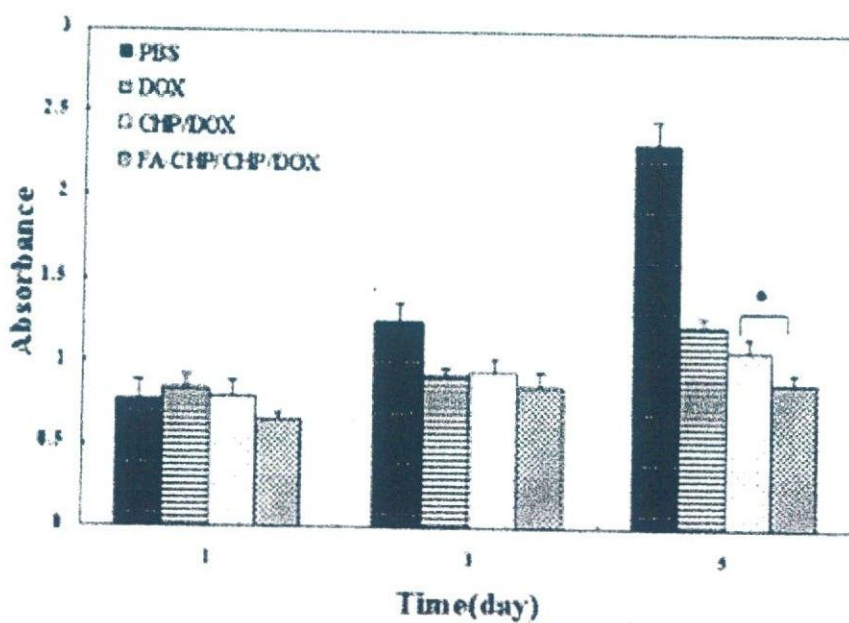


Figure 3. Cytotoxicity of free DOX, CHP/DOX complexes and FA-CHP/CHP/DOX complexes toward KB cells in a folate-depleted medium ($p < 0.05$).

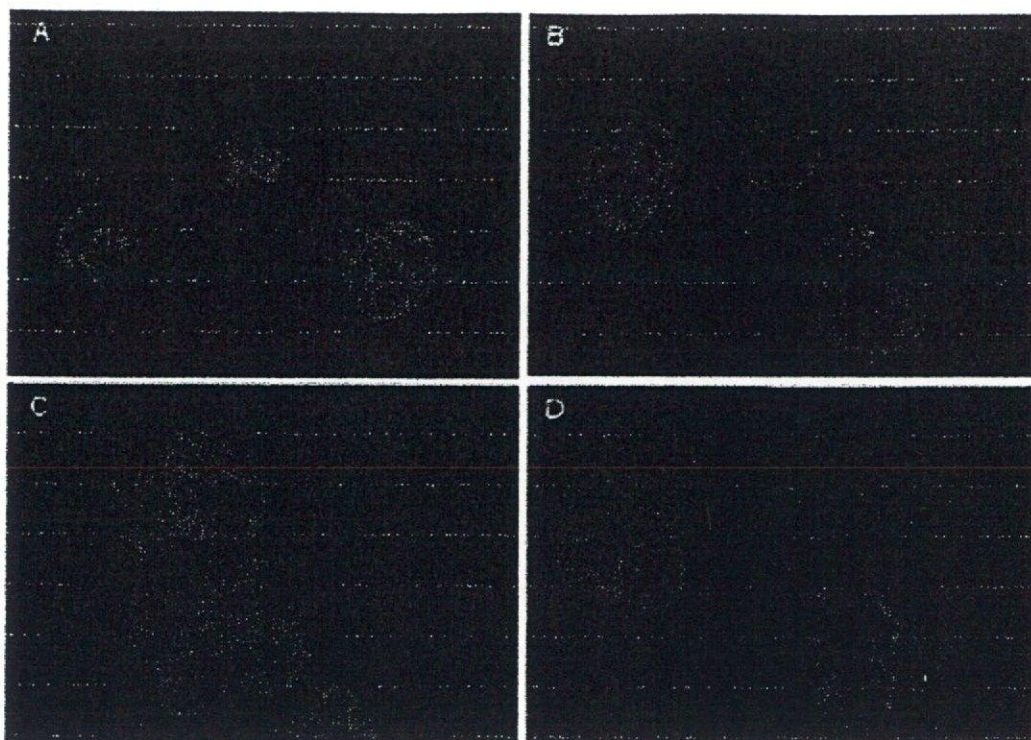


Figure 4. Confocal microscopic images of KB cells. (A) Free DOX; (B) CHP/DOX complexes; (C) FA-CHP/CHP/DOX complexes in a folate-depleted medium; and (D) FA-CHP/CHP/DOX complexes in a folate-overdosed medium.

folate-overdosed medium, suggesting that FA-CHP/DOX were taken up by a FR-mediated endocytosis pathway, because it is known that endocytosis via FR can be blocked by a high concentration of free folic acid in the medium [16]. In contrast to free DOX and CHP/DOX, it is thought that FA-CHP/DOX is mainly distributed to the cytoplasm by a receptor-mediated endocytosis process in this study.

Selective Efficacy of FA-CHP/DOX *In Vivo*

For *in vivo* studies, human tumor xenografted nude mice were used to examine the anti-tumor effects of FA-CHP/DOX. Based on DOX, an equivalent amount of free DOX, CHP/DOX or FA-CHP/DOX was administered intravenously to separate groups of mice. No difference in the growth of each group was noted at day 13 after the initial administration. After day 21, there was a large difference between the CHP/DOX group and the FA-CHP/DOX group (Figure 5). The administration of FA-CHP/DOX was effective, significantly suppressing the tumor growth of KB cells over-expressing FR compared to the free

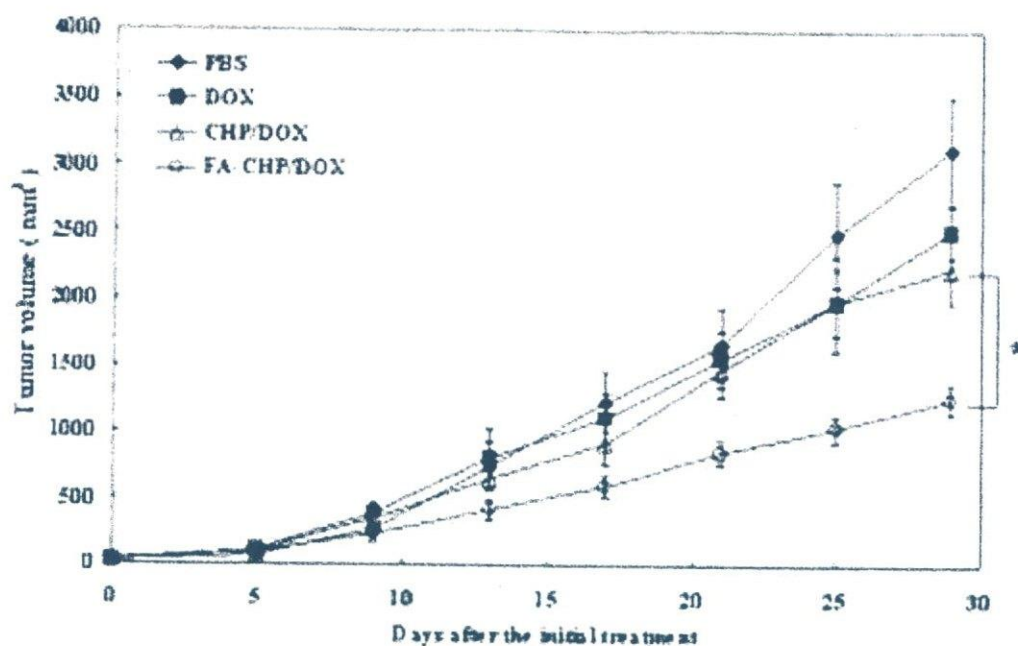


Figure 5. Tumor volumes for free DOX, CHP/DOX complexes and FA-CHP/DOX complexes ($p < 0.05$).

Table 1. Tumor weight differences at day 29 after the initial treatment.

Agents	Weight (g)
PBS	3.01 ± 0.26
DOX	2.35 ± 0.50
CHP/DOX	1.99 ± 0.34
FA-CHP/DOX	1.43 ± 0.23

DOX and CHP/DOX groups. The average tumor weight of the FA-CHP/DOX group was about 40% less than that of the free DOX group (Table 1). Moreover, there were no serious side effects such as weight loss in the present study (data not shown). The effect of CHP derivatives on cancer cells has been attributed to the active targeting of tumor cells [2]. However, in the present study, the effect of FA-CHP/DOX on delayed tumor volume growth may additionally be attributed to passive targeting mechanisms such as the enhanced permeation and retention (EPR) effect of a nano-sized drug delivery system [17]. In the growing tumor tissues, then release the growth factor (VEGF) is released to facilitate neo-vascularization. As a result, new vessels are formed, but their cell junctions are not as tight as those of normal tissues. Vascular permeability in tumors is important

in the delivery of macromolecular anti-cancer agents and the low clearance of macromolecules from the tumor is attributed to the fact that they remain in the interstitial tissue of the tumor for a long time [17]. The effect on delayed tumor volume growth found in this study might be correlated with a repeated administration (every fourth day for six doses).

Taken together, these results suggest that FA-CHP could serve as a targeted agent against tumors over-expressing FR. Further detailed tissue distribution and pharmacokinetic studies of FA-CHP in mice, however, must be investigated, because FR- α is expressed in the proximal tubules of the kidney, and therefore, will bind to receptor-targeted agents that are filtrated from the blood stream. In whole-body imaging of FR-positive tissues using folate-radionuclide conjugates, the only normal tissues recognized by the probe were the kidney and peripheral blood macrophages [18,19].

CONCLUSIONS

FA-CHP alone has no cytotoxic activity against cancer cells. However, the formulation of a mixture of FA-CHP/CHP/DOX significantly inhibited the growth of KB cells in folate-depleted DMEM. The cellular uptake of FA-CHP/DOX was evaluated by confocal microscopy. For FA-CHP/DOX, more fluorescently labeled cells were clearly visualized in folate-depleted medium than in folate-overdosed medium.

In *in vivo* studies, the tumor growth of KB xenografted nude mice was suppressed more effectively and selectively by FA-CHP/DOX than by CHP/DOX or free DOX. According to our studies, FA-CHP is an effective vehicle for the delivery of anticancer drugs and has a potential application in the treatment of FR over-expressing tumors.

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Comparison between bioartificial and artificial liver for the treatment of acute liver failure in pigs

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Abstract

AIM: To characterize and evaluate the therapeutic efficacy of bioartificial liver (BAL) as compared to that of continuous hemodiafiltration (CHDF) with plasma exchange (PE), which is the current standard therapy for fulminant hepatic failure (FHF) in Japan.

METHODS: Pigs with hepatic devascularization were divided into three groups: (1) a non-treatment group (NT; $n = 4$); (2) a BAL treatment group (BAL; $n = 4$), (3) a PE + CHDF treatment group using 1.5 L of normal porcine plasma with CHDF (PE + CHDF, $n = 4$). Our BAL system consisted of a hollow fiber module with 0.2 μm pores and 1×10^{10} of microcarrier-attached hepatocytes inoculated into the extra-fiber space. Each treatment was initiated 4 h after hepatic devascularization.

RESULTS: The pigs in the BAL and the PE + CHDF groups survived longer than those in the NT group. The elimination capacity of blood ammonia by both BAL and PE + CHDF was significantly higher than that in NT. Aromatic amino acids (AAA) were selectively eliminated by BAL, whereas both AAA and branched chain amino acids, which are beneficial for life, were eliminated by PE + CHDF. Electrolytes maintenance and acid-base balance were better in the PE + CHDF group than that in the BAL group.

CONCLUSION: Our results suggest that PE + CHDF eliminate all factors regardless of benefits, whereas BAL selectively metabolizes toxic factors such as AAA. However since PE + CHDF maintain electrolytes and acid-base balance, a combination therapy of BAL plus CPE + CHDF might be more effective for FHF.

INTRODUCTION

Fulminant hepatic failure (FHF) has been one of the most challenging problems in clinical medicine. Although plasma exchange (PE) is still a main therapeutic modality in Japan, PE was found not to affect mortality in cases of liver failure^[1]. Therefore, recently the combination of continuous PE and continuous hemodiafiltration (CHDF) has been proposed, and its initial clinical results have been encouraging^[2-4]. In fact, however, no studies have compared CHDF with other therapeutic modalities.

We have developed a BAL system utilizing porcine hepatocytes cultured on collagen-coated beads and reported on its efficacy^[5]. Although many studies have reported the efficacy of CPE + CHDF or BAL treatment, little is known about how these two modalities compare with each other.

The purpose of the present study is to compare the therapeutic efficacy of BAL support for ischemic FHF with that of CPE + CHDF treatment in pigs. In addition, a characterization of the benefit of each treatment was attempted.

MATERIALS AND METHODS

All study protocols were reviewed and approved by the University of Nagasaki Research Animal Resources Animal Care Committee and met both institutional and national guidelines.

Hepatocyte isolation

Hepatocytes were harvested from outbred female white pigs weighing 8 to 10 kg, according to the method described by Seglen with our modification^[6]. Briefly, under isoflurane anesthesia, the portal vein was cannulated with a silicon tube and the liver was perfused *in situ* with 4 L of oxygenated 2 mmol/L ethylenediaminetetraacetic acid

(EDTA) solution at the rate of 400 mL/min at 37°C. Immediately after the perfusion, the liver was removed and placed in a sterile basin. Ten minutes later, the EDTA solution was replaced with a Ca²⁺-enriched Leffert's buffer solution containing 0.05% type P collagenase (Sigma Chemical Co. St. Louis, MO). The solution was re-circulated through the liver in the basin at a rate of 400 mL/min for 20 min under a clean air-filtered hood. After the collagenase perfusion, the digested liver was manually disrupted in cold DMEM (Nissui Pharmaceutical, Tokyo, Japan) containing 10% heat-inactivated fetal calf serum (FCS, Pharmacia LKB Biotechnology, Piscataway, NJ) and 1% penicillin-streptomycin (Life Technologies, Grand Island, NY). The crude liver cells were filtered through a Mesh Filtration Unit (mesh space, 105 μm; Spectrum, Laguna Hills, CA), and the enriched fractions of the hepatocytes were separated by low-speed centrifugation (at 50 g for 2 min, at 4°C). Centrifugation was performed three times. The initial viability of the hepatocytes was determined using the trypan blue dye exclusion test.

Preparation of the bioreactor

Isolated hepatocytes with a viability of better than 90% and collagen coated dextran microcarrier beads (Cytodex 3, Pharmacia LKB Biotechnology, Piscataway, NJ) were incubated with DMEM with 10% FCS in a cell culture bag (Si culture bag, Wako, Tokyo, Japan) for 4 h at 95% air and 5% CO₂ at 37°C. After washing with normal saline, ten billion microcarrier-induced hepatocytes were inoculated into the extra-fiber space (185 mL) of the hollow fiber module with 0.8 m² of total external fiber surface (Plasmaflo OP-08, Asahi Medical, Tokyo, Japan). The hollow fibers were made from polyethylene materials with an internal diameter of 340 μm, a pore size of 0.2 μm, and membrane thickness of 50 μm.

Animal model of fulminant hepatic failure

Under general anesthesia with mechanical ventilation via an endotracheal tube, pigs (weighing 27 to 30 kg) underwent hepatic devascularization consisting of dissection of the biliary tract, hepatic arteries, and ligaments after creation of an end-to-side portocaval shunt according to the method described by Mazziotti *et al.*^[7]. This model was reported to reproduce the many pictures of FHF including, encephalopathy, coagulopathy and brain edema. A femoral artery was cannulated for sampling blood and monitoring blood pressure. A jugular vein was also cannulated with a double lumen catheter for blood access. The volume of the replacement fluid was adjusted over the range of 3-6 L, with 5% dextrose lactate Ringer solution depending on the response of the FHF pigs.

Circulation system of BAL

The BAL system consisted of a hollow fiber module inoculated with microcarrier-attached porcine hepatocytes, a plasmapheresis unit (Plasauto 2500 and Plasmaflo OP-2, Asahi Medical, Tokyo, Japan), a roller pump, and a plasma reservoir (Figure 1). The system was oxygenated and warmed at 37°C using a membrane-type oxygenator. Whole blood was removed at a rate of 35-45 mL/min from the jugular vein and was separated to plasma at a

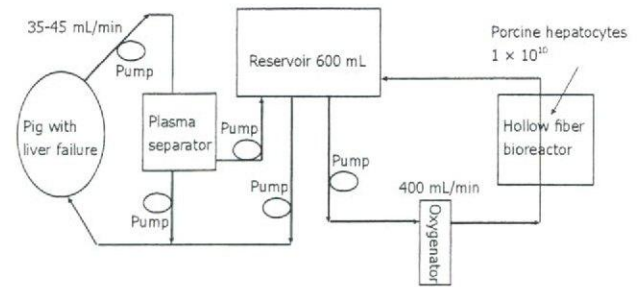


Figure 1 Schematic drawing of our bioartificial liver (BAL) system. It consists of 1 × 10¹⁰ cultured allogeneic hepatocytes in the outer space of a hollow fiber.

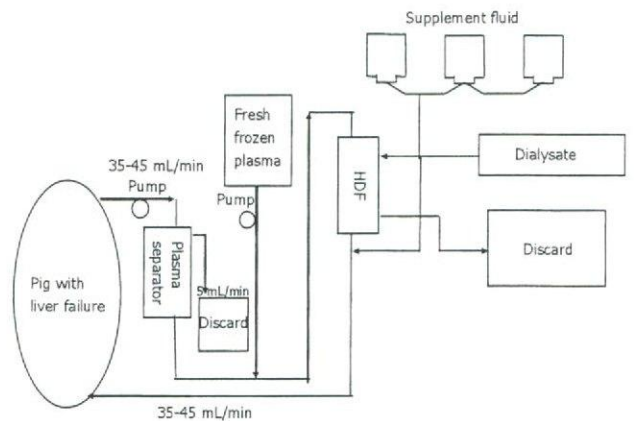


Figure 2 Schematic drawing of continuous plasma exchange (CPE) with allogeneic plasma and continuous hemodiafiltration system (CHDF).

rate of 15 mL/min. The separated plasma was perfused through the bioreactor at a rate of 400 mL/min and was recirculated through the whole system with the transmission reservoir (Figure 1).

Circulation system of PE + CHDF

The PE + CHDF system consisted of the plasmapheresis unit mentioned above, a high performance hemodiafiltration unit (Panflo, APF-03S, Asahi Medical, Tokyo, Japan) and three roller pumps (Figure 2). In this system, the apparatuses were connected in a series. PE + CHDF was performed for 6 h. An average of 1.5 L fresh porcine frozen plasma was used during PE treatment. Dialysis was performed concomitantly at a flow rate of 50 mL/min using a dialysis fluid containing bicarbonate buffer (Sublood B; Fuso Pharmaceutical, Tokyo, Japan).

Experimental design

Animals undergoing hepatic devascularization were divided into three groups as follows: a non-treatment group (NT; only plasma separation, *n* = 4), a BAL treatment group (BAL; *n* = 4), and a CPE + CHDF treatment group (CPE + CHDF; *n* = 4). The BAL or CPE + CHDF treatments were initiated at 4 h after completion of hepatic devascularization. The pigs in both the BAL and CPE + CHDF groups were treated for 6 h and were given 800 U/h of heparin as an anticoagulant. After 6 h BAL or CPE + CHDF was stopped and the pigs were observed until their death.

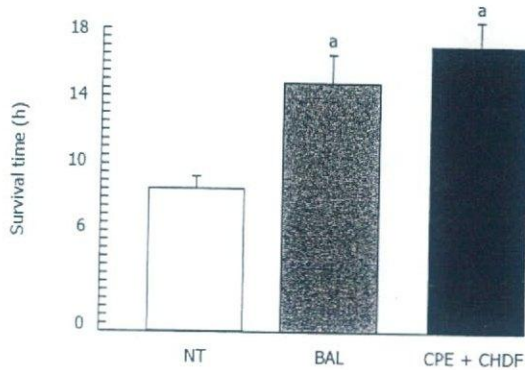


Figure 3 Survival after induction of hepatic failure. * $P < 0.05$ vs NT group

Assessments

Conventional liver function tests including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), the plasma levels of branched chain amino acids (BCAA), aromatic amino acids (AAA), the ratios of the BCAA to the AAA (Fischer's ratio) after each treatment, and the changes in plasma ammonia were determined. Plasma levels of electrolytes, blood urea nitrogen (BUN), creatinine, and pH were measured. Survival time was also observed.

Statistical analysis

Data were expressed as mean \pm SD, and were analyzed statistically using a non-parametric test. $P < 0.05$ was considered to indicate a significant difference.

RESULTS

Survival

Although the pigs in the BAL and the CPE + CHDF groups survived significantly longer than those in the NT groups, there were no significant differences between the BAL group and the CPE + CHDF group (Figure 3; control: 10.3 ± 1.1 h; BAL: 18.0 ± 1.3 h; CPE + CHDF: 19.2 ± 0.7 h).

Liver functions

Plasma levels of AST and ALT showed no significant differences among the three experimental groups. The Fischer's ratio was highest in the BAL group (NT: 0.8 ± 0.1 ; BAL: 1.5 ± 0.3 ; CPE + CHDF: 1.1 ± 0.2). In the NT group, plasma levels of both AAA and BCAA increased gradually during the treatments. In the BAL groups, whereas AAA levels in plasma decreased significantly, BCAA levels remained unchanged. In contrast, in the CPE + CHDF group, both AAA and BCAA levels in plasma decreased during the treatments (Figure 4).

The elimination capacity of ammonia in both the BAL and CPE + CHDF groups was significantly higher than that in the NT group, but there were no significant differences between the BAL and CPE + CHDF groups (Figure 5).

Electrolytes and pH

As for electrolyte status, serum potassium level increased

with time in both the NT and BAL group. In the CPE + CHDF group, the increase in potassium level was kept within the normal range (Figure 6). As for acid-base balance acidosis deteriorated with time in the NT and BAL group but was rather maintained in the CPE + CHDF group (Figure 6). Although the same pattern among the three groups was also seen, there were no statistical differences between the groups.

DISCUSSION

Although most nonbiological or biological liver support systems rely on blood detoxification, the removal of medium-sized molecules, which cause hepatic coma, does not improve the survival rate of patients with severe encephalopathy^[1]. This implies that the replacement of biotransformation and of liver synthetic functions is also needed in order to maintain life. At present, only intact hepatocytes seem to be able to achieve it^[8-10].

In this experiment, the most striking findings for BAL were its effects on changes in plasma ammonia and on amino acids. The elimination capacity of plasma ammonia in BAL and CPE + CHDF was significantly higher than that in NT, although there were no significant differences between BAL and CPE + CHDF. These results indicated that hepatocytes in BAL functioned well and that its ammonia elimination capacity was not inferior to that of the CPE + CHDF treatment. The improvement of Fischer's ratio in BAL treatment was attributable to the decrease in AAA, which is one of the toxic substances in hepatic failure and which is metabolized by intact hepatocytes^[11]. It is noteworthy that AAA was selectively eliminated in BAL, whereas both AAA and BCAA, which are beneficial amino acids, were eliminated in CPE + CHDF. This implies that CPE + CHDF eliminated all factors regardless of toxicity or necessity, whereas BAL selectively metabolized toxic factors such as AAA in the blood. To the best of our knowledge, the present results are the first to report the characteristics of two different liver support systems *in vivo*.

However, as for the maintenance of acid-base balance and electrolytes, CPE + CHDF was superior to BAL. That is, in the model of FHF in pigs, CPE + CHDF outperformed BAL in the maintenance of general status such as acid-base balance and electrolytes abnormalities. In addition, although there were no significant differences between these two groups, the pigs in the CPE + CHDF group survived slightly longer than those in the BAL group. We attributed this to better renal functions and maintenance of acid-base balance in the CPE + CHDF group.

Also, it was possible that CPE + CHDF removed all growth factors that might be needed for a diseased liver to regenerate. This kind of nonselective removal of the indispensable factors has been reported previously by researchers, including ourselves^[12]. We showed that, in the rat model undergoing partial hepatectomy, blood exchange therapy reduced the serum level of hepatocyte growth factor significantly, resulting in better liver regeneration with a PCNA labeling index. In this sense, BAL might be valuable in light of its bio-transforming activity, which does not remove all factors needed.

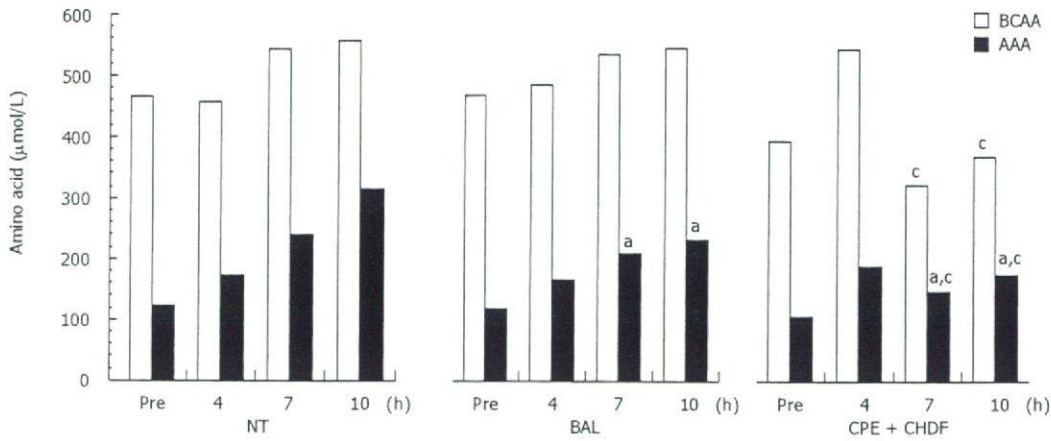


Figure 4 Time transition of the levels of amino acid after induction of acute liver failure in (A) NT group, (B) BAL group and (C) CPE+CHDF group. ^a*P* < 0.05 vs NT group, ^c*P* < 0.05 vs NT group and BAL group.

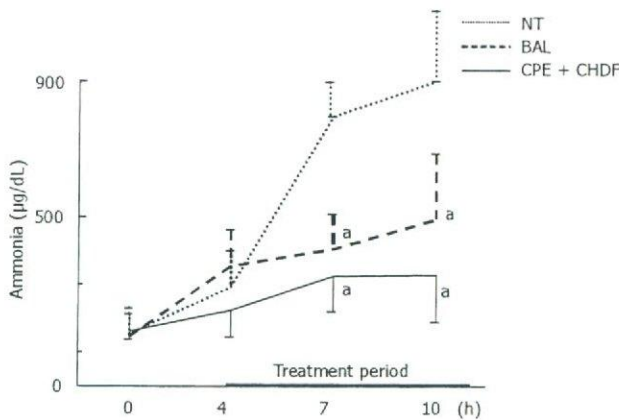


Figure 5 Time transition of the levels of ammonia in the blood after induction of acute liver failure. ^a*P* < 0.05 vs NT group.

As to survival time, we did not find the difference between the BAL and CPE + CHDF, although both treatments prolonged the survival of pigs with ischemic liver. Therefore, it would be possible to use other models of liver failure such as the one recently published, since our model of liver failure was too aggressive with no chance of spontaneous liver regeneration^[13].

In conclusion, we have compared the BAL and CPE + CHDF treatments, especially concerning their elimination capacity. We expect that the combination of BAL and CPE + CHDF would serve as an effective bridging therapy to liver transplantation or liver regeneration in patients with liver failure.

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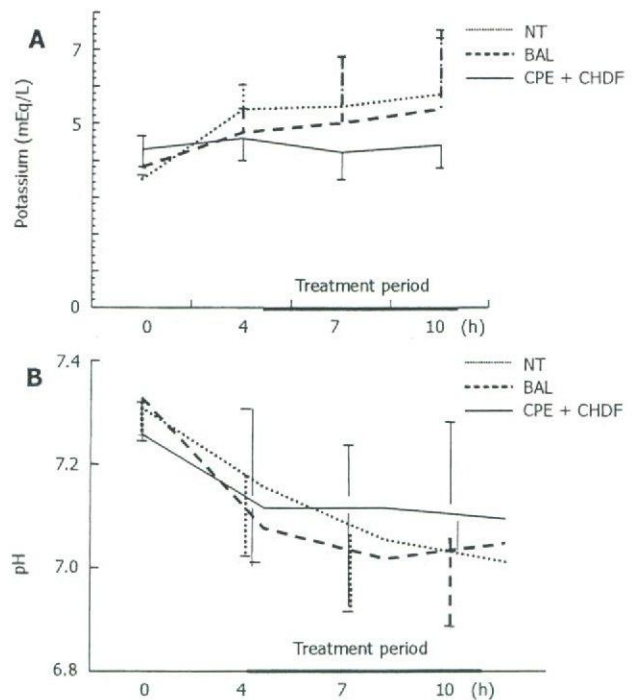


Figure 6 Time transition of the serum levels of (A) potassium and (B) pH after induction of acute liver failure.

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Total Vascular Exclusion Safely Facilitates Liver Specific Gene Transfer by the HVJ (Sendai Virus)-Liposome Method in Rats

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Background. Most virus mediated transfection systems are efficient; however, their highly immunogenic properties do tend to cause clinical problems. HVJ-liposome vector is a hybrid vector consisting of liposome and inactivated sendai virus (hemagglutinating virus of Japan HVJ), which has been reported to be have a low immunogenicity, while it can also be repeatedly administered. To enhance the transfection efficiency, especially in the liver, we investigated the efficacy of total vascular exclusion (TVE) during the portal vein injection (PVI) of the vectors.

Materials and methods. β -galactosidase and luciferase expression were used as reporter genes. Wistar rats were injected with HVJ-liposome through PVI without TVE (PVI group, $n = 10$) or PVI with TVE (PVI + TVE group, $n = 10$). The control rats were infused with equal volumes of saline through the portal vein (control group $n = 12$). The transfection efficiencies were assessed by β -galactosidase staining and a luciferase assay. Biochemical and histological analyses were performed to evaluate the tissue toxicity after gene transfer.

Results. The reporter genes expression in the liver dramatically increased after PVI + TVE in comparison to after PVI alone (1.2×10^5 versus 1.5×10^4 RLU/mg protein, $P < 0.05$ according to a luciferase assay). Notably, the extrahepatic “leaky” transgene expression could be minimized by PVI + TVE, whereas the general condition remained unchanged according to both the biochemical parameters and histological findings.

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Conclusions. The present data indicate that PVI + TVE may thus facilitate the liver-specific gene delivery using the HVJ-liposome method and this modality might also be applicable to other gene transfer systems. © 2006

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Key Words: gene transfer; rat liver; HVJ-liposome.

INTRODUCTION

Gene therapy has a considerable therapeutic potential and it may be useful as a novel approach for the treatment of a variety of diseases [1–3]. The liver is the most important target organ where many genetic disorders as well as acquired diseases, such as hepatitis and primary or metastatic cancer, are manifested [4]. Advancements in hepatic gene therapy depend (to a large degree) on the development of delivery systems to the liver. Among the numerous techniques that have been developed thus far to introduce genetic material into the liver, viral vectors have the most powerful gene transfer activity, however, all such methods have certain limitations at present. For example, retrovirus vector was first developed and well characterized, but it requires the division of the target cell. *In vivo* gene transfer using retrovirus needs an enhanced proliferation of the hepatocytes by either a partial hepatectomy or growth factor treatment. Adenoviruses can be delivered at a high frequency into the liver, and then are able to infect non-dividing hepatic parenchymal cells; however, they induce a strong immunological response because of its highly immunogenic viral proteins, thus resulting in the transient nature of transgene expression. Despite genetic modifications to reduce the immunogenicity of adenoviral vectors, whose efficacy for long-term gene therapy remains to be elucidated, the

use of long-term gene expression still has to be assessed. Other viral vectors, such as herpes virus, lentivirus, and adeno-associated virus also have potential limitations in their clinical application [5-7]. Therefore, non-viral gene transfer techniques, including the injection of naked plasmid DNA, and liposome mediated DNA delivery, have been developed [8, 9].

Under these circumstances, a hybrid vector has been developed that involves the entrapment of DNA and a non-histon chromosomal protein within liposomes and the use of the Sendai virus (HVJ, hemagglutinating virus of Japan) to enhance the fusion of the liposome and the target cell membrane [10]. The Sendai virus is inactivated before use and only its fusiogenic-envelope protein is used for the development, thereby eliminating the possibility of contaminating an individual with a wild type virus. The Sendai virus has already been demonstrated to have a potential to infect a variety of organs, in other words, the mode of transfection mediated by the Sendai virus is not tissue specific. Although the infectivity of the HVJ-liposome to the liver has already been proven [11, 12], the systemic administration of HVJ-liposome via the intravenous route resulted in a systemic transgene expression. To facilitate the usefulness of the HVJ-liposome for hepatic gene therapy, we evaluated the efficacy of the total vascular exclusion technique (TVE) during gene transfer vector injection regarding its transfection efficiency in the liver.

In the present study, we analyzed the efficiency, tissue specificity, and safety of liver-directed gene transfer by portal vein injection of HVJ-liposome under TVE in a rat model.

MATERIALS AND METHODS

Animals

Outbred male Wistar rats, weighing 200 to 250 g (CRJ Charles River Japan, Kanagawa, Japan) were used in the present experiments. These animals were kept under standard laboratory conditions of alternating 12-h periods of light and darkness and a standard laboratory diet. All of the studies were performed according to the rules of the University of Nagasaki Research Animal Resources Guidelines.

Reporter Genes

We used the bacterial LacZ gene and firefly luciferase gene as reporter genes. The lacZ gene was selected so that we could detect transfected cells by highly sensitive *in situ* LacZ staining method. To quantitate the level of transgene expression, we used a luciferase assay system, as reported previously [13]. The LacZ gene used in this study was a pSV- β -galactosidase vector (β -gal) (Promega, Madison, WI), in which the bacterial lacZ gene was driven by the simian virus 40 (SV40)-early promoter/enhancer. As a reporter firefly luciferase vector, we used the PGL2-control vector (Promega) where the firefly luciferase gene was driven by the SV40-early promoter/enhancer. These plasmids were amplified in *Escherichia coli* and purified using the Qiagen plasmid DNA isolation kit (Chatsworth, CA).

Preparation of HVJ-Liposome

HVJ-liposome was prepared as described previously [10, 12, 14, 15]. Briefly, phosphatidylserine (Avanti polar), phosphatidylcholine (Sigma Chemical Co., St. Louis, MO), and cholesterol (Sigma) were mixed at a weight ratio of 1:4.8:2. The lipid mixture (10 mg) was dried by removing the chloroform, and then it was hydrated in 200 μ l balanced salt solution (BSS 137 mM NaCl, 5.4 mM KCl, 13 mM Tris-HCl, pH 7.6) containing a DNA-high mobility group I (HMG-I) complex (200 μ g:64 μ g), which had been incubated previously at 20°C for 1 h. The mixture was intensely agitated by vortexing for 30 s and then it was left to stand for 30 s. This procedure was repeated eight times. Purified HVJ was inactivated by ultraviolet irradiation (1980 J/m²) and 15,000 hemagglutinating units (HAU) HVJ mixed with a DNA-HMG-I-liposome suspension. The mixture was incubated at 4°C for 10 min and then for 1 h at 37°C while shaking at 120/min. Free HVJ was removed from the HVJ-liposome solution by gradient centrifugation of the sucrose density. The second layer of sucrose containing the HVJ-liposome was thereafter collected.

Surgical Procedure for *In Vivo* Gene Transfer

Figure 1 illustrates how to tranfect the reporter genes to the liver by HVJ-liposome under total vascular exclusion (TVE). Briefly, under ether anesthesia, an abdominal midline incision was made, and the liver was isolated from the surrounding tissues. Next, the supra hepatic vena cava just beneath the diaphragm (SHVC), and infrahepatic vena cava above the right kidney (IHVC), hepatic artery (HA) was clamped, followed by the portal vein injection of HVJ-liposome containing 20 μ g of DNA at a volume of 1 ml with a clamping time of 5 min (PVI + TVE group $n = 10$). During this procedure, the portal vein was clamped at the periphery of the injection site. The control rats were infused with equal volumes of saline through the portal vein (control group $n = 12$). The other rats were infused with HVJ-liposome containing 20 μ g DNA via portal vein without TVE (PVI group $n = 10$). Liver biopsies were performed from each lobe of the rats at 1, 3, 7, 14, 21 days post-transfection. All animals tolerated these surgical procedures well during the observation period.

Luciferase Assay

Liver samples were obtained at 1, 3, 7, 14, 21 days after transfection. These tissue specimens were diluted by 3 \times reporter lysis buffer

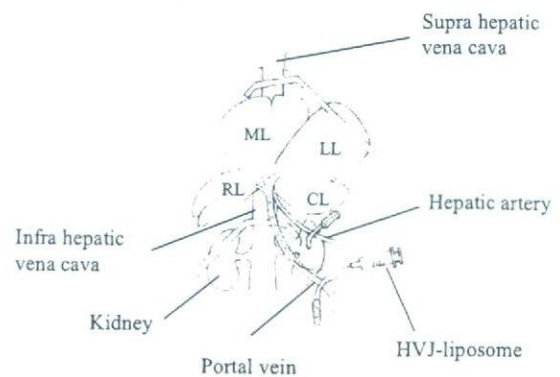


FIG. 1. Procedure of total liver exclusion during HVJ-liposome mediated gene transfer into rat liver *in vivo*. During 3-min clamping of supra- and infra-hepatic vena cava, portal vein, and hepatic artery, HVJ-liposome solution was infused selectively into the liver through inserted catheter at a flow rate of 1.0 ml/min (total 1.0 ml). Abbreviations: RL, right lobe; ML, middle lobe; LL, left lobe; CL, caudate lobe.

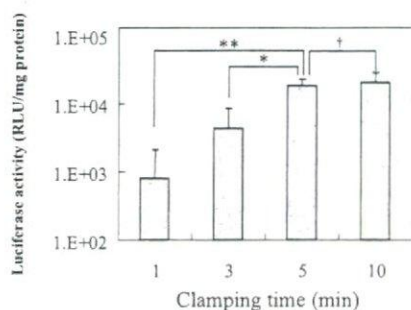


FIG. 2. Optimum clamping time of TVE. Luciferase gene (15 μ g) was introduced into the portal vein under 1, 3, 5, 10 min of TVE. Transgene expression in the liver became maximum at 5 min of TVE. * $P < 0.05$, ** $P < 0.01$, †not significant.

supplied from a kit (Promega). Aliquots were assayed for luciferase activity in a Lumat LB9501 luminometer (Berthold Systems, Alquiippa) using 20 μ l of supernatant and 100 μ l of reconstituted luciferase assay reagent (Promega). The light units produced were measured, starting 3 to 5 min after mixing, for 20 s. The results were expressed in relative light units, or the actual luciferase units as corrected based on the protein content as determined by the Bradford method [16].

β -Gal Histochemistry

To evaluate the transfection efficiency, the animals were sacrificed and the whole liver was removed at the indicated time points. These tissue specimens were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde for 3 h at 4°C, and then were rinsed with phosphate-buffered saline (PBS) three times. To detect the expression of β -galactosidase, the fixed tissues were stained with X-gal (Nakarai, Kyoto, Japan) for 6 h and then were counterstained by hematoxylin, as described previously [12]. The transfection efficiencies were evaluated by counting blue-stained cells in 10 randomly

selected fields under magnification $\times 100$ followed by using the NIH image Macintosh software package.

Assessment of Toxicities

As a parameter of systemic injury, we measured serum values of aspartate aminotransferase (AST), alanine transaminase (ALT), total bilirubin (T-Bil.), blood urea nitrogen (BUN), creatinine (Cr.), and lactate dehydrogenase (LDH) at 48 h after transfection using an automated analyzing system. To evaluate the pathologic changes in the liver tissue specimens, formalin-fixed, paraffin-embedded tissues were sectioned, and thereafter were stained by hematoxylin and eosin (H&E).

Statistical Analysis

Data are presented as the mean \pm standard deviation. Statistical analyses were performed using the Mann-Whitney U test. A value of $P < 0.05$ was defined as significant.

RESULTS

Optimal Clamping Time of TVE to Maximize the Transgene Expression

To examine the optimum clamping time of TVE, luciferase gene (15 μ g) was introduced into the portal vein under 1, 3, 5, 10 min of TVE ($n = 5$ each). As shown in Fig. 2, the transgene expression in the liver reached a maximum at 5 min of TVE. Therefore, we selected 5 min as a clamping time for following experiments.

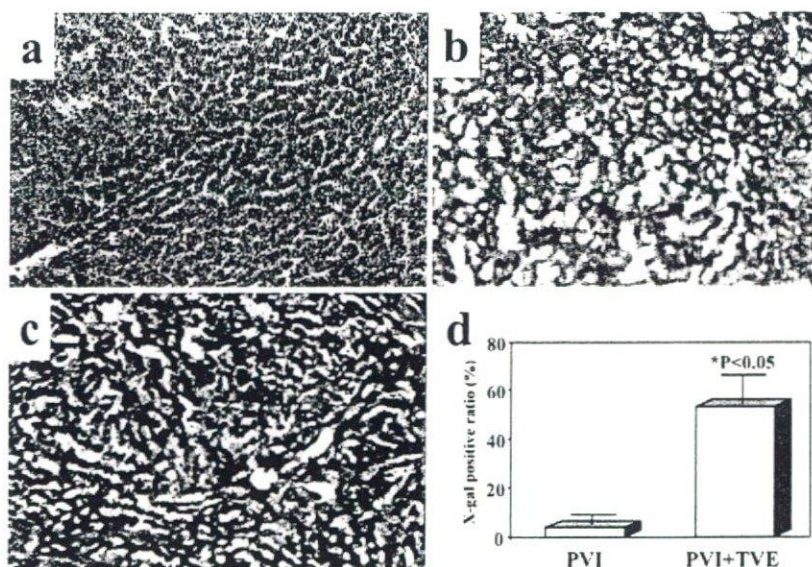


FIG. 3. Detection of transgene expression by X-gal histochemistry of rat liver. Blue stained cells expressed *E. coli* β -galactosidase. β -gal positive cells were detected in the β -gal-HVJ liposome injected rat liver (B), but not in saline injected liver (A). More than 50% of hepatocytes were positively stained for β -gal by HVJ-liposome mediated gene transfer under TVE (C, $\times 200$). A statistically significant difference was observed between the PVI and PVI + TVE group (D). (Color version of figure is available online.)