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In Vivo Liver-Directed Gene Transfer in Rats and Pigs with Large Anionic Multilamellar Liposomes: Routes of Administration and Effects of Surgical Manipulations on Transfection Efficiency

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Conventional large (500–800 nm) multilamellar liposomes encapsulating DNA have been used *in vivo* as gene vectors into rat and pig liver. By using the *intraportal vein route*, high dose DNA (10 mg/kg) provided low efficiency and transient luciferase gene expression in the liver. This gene expression was, however, increased by liver resection (> 50%), ischemia (20 min) or orthotopic transplantation. As evidenced by histochemical analysis of β-galactosidase expression, the gene transfection mainly ensued in Kupffer cells, but spleen and lung were contaminated. In comparison, injection *into the bile duct* of even 25-fold lower dose of liposome-encapsulated DNA (0.4 mg/kg) produced higher (100-fold) and long-lasting (during 6 days, at least) luciferase expression in rat liver. The gene expression was restricted to the liver and enhanced by liver resection. By this route, transgene-expressing cells were mainly hepatocytes. A treatment with colchicine prior to the administration of the vector allowed the persistence of relative high gene expression for at least 7 days. In pigs, qualitatively similar, but quantitatively less efficient gene expression was obtained by either the portal vein or the bile duct route. These results indicate that intrabile duct route might render large non-viral vectors applicable to gene transfer into the hepatocytes. The efficiency of liposome-mediated gene transfer into the liver can be increased by liver resection, ischemia or transplantation performed before DNA injection.

Keywords: Non-viral vector, Portal vein, Bile duct, Hepatectomy, Liver transplantation

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INTRODUCTION

The development of efficient gene transfer into the liver could prevent the onset or progression of several liver diseases such as hepatitis, primary and metastatic liver cancers, genetic defects or, in liver transplantation acute cellular rejection. For this purpose, viral vectors have been widely studied and have demonstrated high and long-lasting transfection efficiency. However, these viral carriers have several disadvantages. Retroviral vectors require active cell division for integration of viral DNA into the host genome (Miller *et al.*, 1990) and induce a cytotoxic immune response against the infected cells (Izembart *et al.*, 1999). Adenoviral vectors also elicit a host immune response and induce acute inflammation (Sullivan *et al.*, 1997; Yang *et al.*, 1994), nonetheless, "gutless" adenoviral vectors are now under investigation (Chen *et al.*, 1997). Adeno-associated viral vectors might be safe, but the current purification procedure is cumbersome and results in small quantities of virus (Linden and Woo, 1999).

In contrast, non-viral vectors such as liposomes-encapsulated (Len) DNA, cationic lipids–DNA complexes and DNA-polylysine particles could overcome these problems, but gene expression induced by these vectors is often low after intravascular injection *in vivo*. The major causes of such a low expression are the rapid uptake of vectors by reticuloendothelial system including Kupffer cells and the DNA degradation in the lysosomes even if vectors enter hepatocytes (Pouton and Seymour, 1998). To circumvent this second limitation, endosomolysis by adenoviral capsids or lysosome-disrupting peptide have been investigated (Cristiano *et al.*, 1993; Baru *et al.*, 1998). Although the level of gene expression by these methods can be greatly improved in primary hepatocyte cultures (Cristiano *et al.*, 1993), those were limited *in vivo* probably because of an increase of the size of the complexes. Since the diameter of the liver endothelium fenestrations is only less than 150 nm (Wisse *et al.*, 1985), the large size of complexes may limit access to hepatocytes when these are injected intravascularily.

One possible way to overcome these limitations could be to deliver these complexes through the biliary tract. Since the diameter of the bile canaliculi is a few micrometers (Stieger and Landmann, 1996), we hypothesized that large molecules, up to this size, could reach hepatocytes. Furthermore, undesirable uptake by reticuloendothelial cells could be avoided in this administration route. In the present study, we used conventional large multilamellar liposomes as a DNA carrier and compared two different routes of administration, namely intraportal vein and intrabile duct injection in rat and pig models. In addition, the effects on liver gene transfection of several hepatic manipulations, such as partial hepatectomy (PHX), liver ischemia and orthotopic liver transplantation (OLT), were also evaluated, since little is known of such manipulations on gene transfer efficiency.

MATERIALS AND METHODS

Plasmids

The pCI-luc and the pCMVβ plasmids express a firefly luciferase and a bacterial β-galactosidase from a human cytomegalovirus immediately early promoter. The pCMVβ was purchased from Clontech. The pCI-luc was constructed by insertion of the firefly luciferase gene (Xho I-Xba I fragment of pGL3-basic (Promega)) into Xho I and Xba I sites in pCI plasmid (Promega). The pCI-luc-PRE was constructed by insertion of the posttranscriptional regulatory element (PRE) of the hepatitis B virus (Huang and Yen, 1994; 1995) into Not I and Sal I sites in the pCI-luc plasmid. The pCI-luc or the pCI-luc-PRE served for the assessment of transfection efficiency, while the pCMVβ was used in order to determine in which cell type of the liver the transgene was expressed.

Liposome Preparation

Liposomes were prepared by the previously described method (Baru *et al.*, 1995; 1998), with

slight modification. Briefly, to produce 1 g of liposomes composed of egg phosphatidylcholine (EPC; Lipoid KG) and dimyristoyl phosphatidylglycerol (DMPG; Sygena), a tert-butanol solution of EPC and DMPG was prepared by dissolving 0.9086 g EPC and 0.0914 g DMPG in 10 ml tert-butanol (Riedel-de Haen) and 1 ml H_2O. The compound was mixed with plasmid DNA, dissolved in H_2O, at a ratio of 100 : 1 (w/w) lipids to DNA and then freeze dried. Before use, the dry lipids and DNA were hydrated in two stages. First, H_2O was added to 30% of DNA (v/w) and the mixture shaken until a homogenous solution was obtained. The solution was then adjusted to 154 mM NaCl using 200 mM NaCl and filtered through a 15 μm filter to eliminate large aggregates from the solution. This method allows the encapsulation of around 50% of the DNA used in multilamellar liposomes independently of the DNA size (Baru *et al.*, 1995). The mean size of the multilamellar liposomes was between 500 and 800 nm as assessed by a light-scattering particle analyzer (N4 plus, Counter Electronics).

To obtain reduced sized liposomes, the liposome solution was filtered through 0.2 and 0.1 μm polycarbonate filters in a LipoFast Large apparatus (Avestin Inc.) to select liposomes of 170–200 nm.

Animals and Surgical Procedures

Male Wistar rats weighing 150–200 g were used and anesthetized with diethyl ether in all rat studies except in the OLT experiment, in which male Wistar rats weighing 300–350 g were used and anesthetized with isoflurane. In the pig studies, outbred pigs weighing around 12 kg were used and anesthetized with enflurane under tracheal intubation.

In rats, intraportal vein injection was performed manually through the mesenteric vein. For intrabile duct injection, the common bile duct was isolated and cannulated with a polyethylene tube (PE 50; Becton Dickinson Co.). Len DNA or naked DNA were injected retrogradely into the biliary tract through a tube using a peristaltic pump (B. Braun) with a flow rate of 0.3 ml/min, followed by infusion of normal saline for 60 min at a flow rate of 0.02 ml/min. After injection, the tube end was kept in the bile duct and the other end inserted into the duodenum so that bile flowed into the duodenum through the tube. At an indicated time-point after the injection, animals were sacrificed and several organs collected. For assays of serum alanine aminotransferase (ALT), asparate aminotransferase (AST), γ-glutamyltransferase (γ-GTP) and total bilirubin, blood samples were taken by retro-orbital puncture 1 day after Len DNA injection into the bile duct.

To assess the effects of several surgical manipulations on foreign gene expression in the liver, PHX, liver ischemia or OLT were performed and Len DNA was injected 1 day or immediately after these surgeries. Several amounts of PHX were carried out in rats by resection of multiple combinations of liver lobes; 9%: only the caudate lobe, 16%: the lower part of right lateral and the caudate lobes, 32%: only the left lateral lobe, 48%: the left lateral, the lower part of right lateral and the caudate lobes, and 70%: the median and the left lateral lobes. Twenty minutes of total liver ischemia was performed by occlusion of the hepatic artery, the portal vein and the bile duct with a vascular clamp. OLT was performed in syngeneic rats, according to the technique described by Kamada and Calne (1983).

In order to examine the effect of colchicine and PHX performed several days after Len DNA injection, four groups of rats were injected with Len DNA in the bile duct. The first and second groups ($n = 4$ each) were injected intraperitoneally with normal saline as controls. The third and fourth group ($n = 6$ each) received 0.75 mg/kg of colchicine (Sigma) intraperitoneally 1 h prior to Len DNA injection. Six days later, a 70% PHX was performed in the first and third group and a sham operation in the second and fourth group. One day after surgery (7 days after Len DNA injection), animals were sacrificed and liver samples were taken. The luciferase activity was measured both in resected and residual livers.

In pigs, intraportal vein injection was performed manually through the portal vein under laparotomy. One of three pigs received 15 mg of a larger-sized

Len DNA (pCI-luc) and in other two pigs, OLT was carried out by a technique developed in our Laboratory (Oike *et al.*, 2000) before Len DNA injection. Immediately before reperfusion, Len DNA (pCI-luc-PRE) was injected into the portal vein from the site of anastomosis. One pig received 70 mg of a smaller-sized (170–200 nm) Len DNA and another received 133 mg of a larger-sized (500–800 nm) Len DNA. To study the bile duct route, four intact pigs were injected with several amounts of larger-sized Len DNA (pCI-luc-PRE). For intrabile duct injection, the outer side of a 22-gauge intravascular cannula (Baxter) was inserted in the cystic duct. Len DNA was injected through this cannula using a syringe pump (Termo) (flow rate: 2.5 ml/min) and subsequently 12 ml of normal saline during the following 60 min. After injection, the cannula was taken out and a cholecystectomy was performed. At several time-point after injection, liver samples were obtained by ultrasound (Siemens)-guided needle biopsies and, at day 6, animals were sacrificed.

Assay for Luciferase Activity

Extraction of luciferase from organs was performed as previously described (Manthorpe *et al.*, 1993). In brief, collected organs were quickly frozen in liquid nitrogen and pulverized into a fine powder. One hundred milligram of tissue powder were homogenized with a cell culture lysis reagent (500 µl each, Promega). After a 30-min incubation at room temperature, samples were frozen and thawed three times and briefly centrifuged to remove cell debris. The supernatant was separated and stored at −78°C until assay. The luciferase activity in 20 µl lysate was determined by the addition of 100 µl of luciferase assay reagent (Promega) using a luminometer (Turner Designs). Relative light units were converted to picogram of luciferase according to a calibration curve based on recombinant luciferase (Promega) as a standard. A low detection limit in our methods is 2.0×10^{-3} pg/µl of lysate. Protein concentration of the lysate was determined with the BCA protein assay kit (Pierce).

Histochemical Staining of β-Galactosidase

Small pieces of the liver obtained from animals injected with Len DNA (pCMVβ) were frozen in isopentane chilled in liquid nitrogen and sectioned on a cryotome. Following fixation with 0.4% formaldehyde and 0.2% glutaraldehyde, the sections were incubated with a 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal; Sigma) solution (5 mmol/l $K_4Fe(CN)_6$, 5 mmol/l $K_3Fe(CN)_6$, 0.8 mg/ml X-Gal, 2 mmol/l $MgCl_2$) at 37°C for 24 h and counterstained with hematoxylin and eosin.

RESULTS

A. Intraportal Vein Injection in Rats

Gene Transfer into the Whole Liver

Len DNA (pCI-luc or pCI-luc-PRE) (2.5 mg) or naked DNA (pCI-luc-PRE) were injected through the portal vein. No apparent toxicity was observed in animals receiving such high doses of lipids and DNA. However, one day after Len DNA injection, there was only trace of luciferase activity in the liver (pCI-luc ($n = 3$); 0.13 ± 0.1 (mean ± standard error (SE)) and pCI-luc-PRE ($n = 3$); 0.11 ± 0.03 pg/mg of protein). This level was comparable to the level detected in rats injected with naked DNA ($n = 3$) (0.03 ± 0.03 pg/mg of protein; unpaired t test). By three days after injection, the luciferase expression has disappeared (Fig. 1 and Table I).

Gene Transfer after Partial Hepatectomy

Five groups of three rats underwent an intraportal vein injection with 2.5 mg of Len DNA (pCI-luc) 24 h after 9%, 16%, 32%, 48% or 70% partial hepatectomy. As shown in Fig. 2, a PHX of more than 48% increased the transgene expression in the liver 1 day after injection. Seventy percent PHX increased the level of gene expression by seven fold. This increased gene expression was also observed when pCI-luc-PRE was used instead of pCI-luc and when PHX was performed immediately before Len

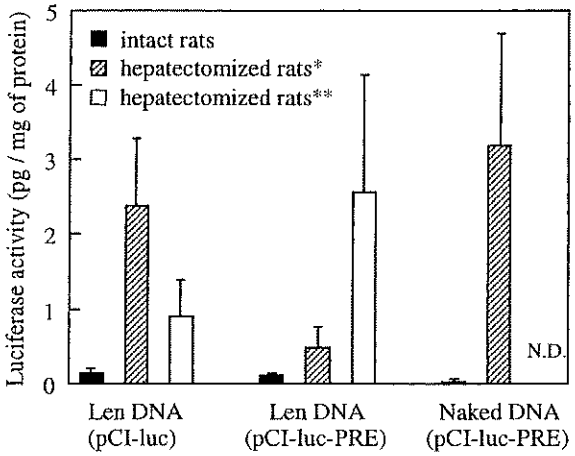

FIGURE 1 Luciferase activity in the liver 1 day after Len DNA injection into the portal vein. Large sized (500–800 nm), negatively charged Len DNA (pCI-luc or pCI-luc-PRE) (2.5 mg) were injected i.v. through the portal vein in 3–5 intact or hepatectomized rats. The same amount of naked DNA (pCI-luc-PRE) was injected and served as control. Extraction of luciferase from the liver and activity measurements were performed as described in Materials and Methods section. The protein concentration in each sample was measured using the BCA protein assay kit and the specific luciferase activity in each sample was calculated. The results are expressed as mean ± SE. *PHX performed immediately before Len DNA or naked DNA injection. **PHX performed 1 day before Len DNA injection. N.D.: not determined.

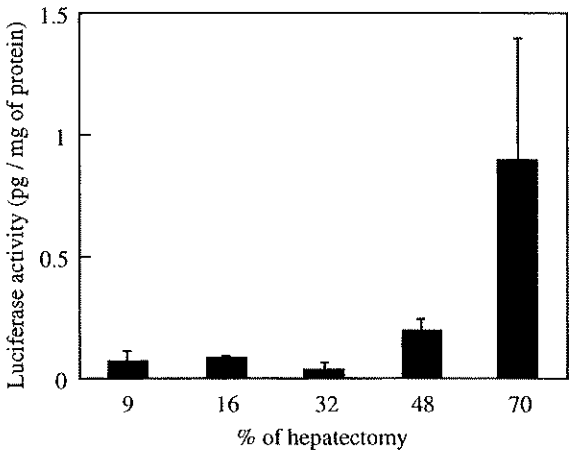

FIGURE 2 Effect of different degrees of hepatectomy on transgene expression in the liver. Large sized (500–800 nm) and negatively charged Len DNA (pCI-luc) (2.5 mg) was injected into the portal vein 1 day after PHX in groups of 3 rats. The specific activity of luciferase in each sample 1 day after Len DNA injection was calculated as described in Fig. 1. The results are expressed as mean ± SE.

TABLE I Time course of transgene expression following large Len DNA injection into the portal vein in rats

	Luciferase activity (pg/mg of protein)		
	Day 1	Day 3	Day 6
Intact rats	0.11 ± 0.03 ($n=3$)	0 ($n=3$)	N.D.
Hepatectomized rats*	0.47 ± 0.3 ($n=5$)	0.08 ± 0.04 ($n=3$)	0 ($n=2$)
Hepatectomized rats†	2.6 ± 1.6 ($n=4$)	0.07 ± 0.03 ($n=3$)	N.D.

Male Wistar rats received 2.5 mg of pCI-luc-PRE encapsulated by liposomes composed of EPC-DMPG. The results were expressed as mean ± SE.
*PHX performed immediately before Len DNA injection;
†PHX performed 1 day before Len DNA injection; N.D.: not determined.

DNA injection (Fig. 1). However, these levels of luciferase activity varied greatly among different animals and were equivalent of those obtained after injection of the same dose of naked DNA (pCI-luc-PRE).

In order to examine the effect of DNA dose, several doses of Len DNA (0.1, 0.5, 0.75, 1.0, 1.5 and 2.0 mg of pCI-luc; $n=2$ each) were administered into the portal vein immediately after 70% PHX. These doses, however, were not sufficient to demonstrate efficient protein expression (0, 0.063, 0.084, 0.053, 0.048 and 0.164 pg/mg of protein, respectively). Different sized liposomes were also tested but smaller-sized liposomes did not provide better transgene expression (data not shown).

After 70% partial hepatectomy, the tissue distribution of gene expression following intraportal vein injection was assessed (Fig. 3). One day after a 2.5 mg Len DNA injection (pCI-luc), the highest luciferase activity was detected in the liver but significant luciferase expression was also evident in both spleen and lung, as previously reported in mice (Baru *et al.*, 1995). In the liver, microscopically, as judged by morphology, the β-galactosidase-expressing cells appeared to be predominantly Kupffer cells (thin cells located in the sinusoids), and rarely hepatocytes, 1 day after injections of 2.5 mg of Len DNA (pCMVβ) in the portal vein immediately after 70% PHX (Fig. 4B). The frequency of

positive cells was low, only 0–1 cells in 10 mm^2 of liver section.

Table I illustrates the time course of luciferase activity in the liver after injection of Len DNA in whole liver, as well as after 70% hepatectomy. Three days after injection of 2.5 mg Len DNA, only trace (< 0.07 pg/mg of protein) of luciferase gene expression could be detected in some animals. No expression was evident 6 days after intraportal injection of Len DNA.

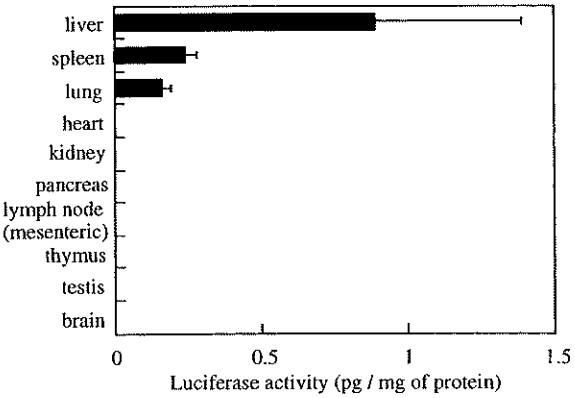

FIGURE 3 Luciferase activity in various tissues 1 day after Len DNA injection into the portal vein in 70% partially hepatectomized rats. Large sized (500–800 nm) and negatively charged Len DNA (pCI-luc) (2.5 mg) was injected in 3 rats 1 day after PHX. Several organs were collected 1 day after Len DNA injection and the specific activity of luciferase in each sample was calculated as described in Fig. 1. The results are expressed as mean ± SE.

Gene Transfer after Ischemia-Reperfusion and Liver Transplantation

Rats undergoing either total liver ischemia ($n = 3$) or OLT ($n = 2$) were injected with 2.5 mg of Len DNA (pCI-luc) immediately after surgery. Both procedures resulted in the increase of luciferase activity in the liver 1 day after injection (2.0 ± 0.7 and 1.6 pg/mg of protein, respectively). In the liver ischemia experiment, however, there was no increased gene expression when Len DNA was injected 30, 60, 90 or 120 min following reperfusion ($n = 3$ each) (0.11 ± 0.02, 0.05 ± 0.03, 0.12 ± 0.05 and 0.16 ± 0.04 pg/mg of protein, respectively).

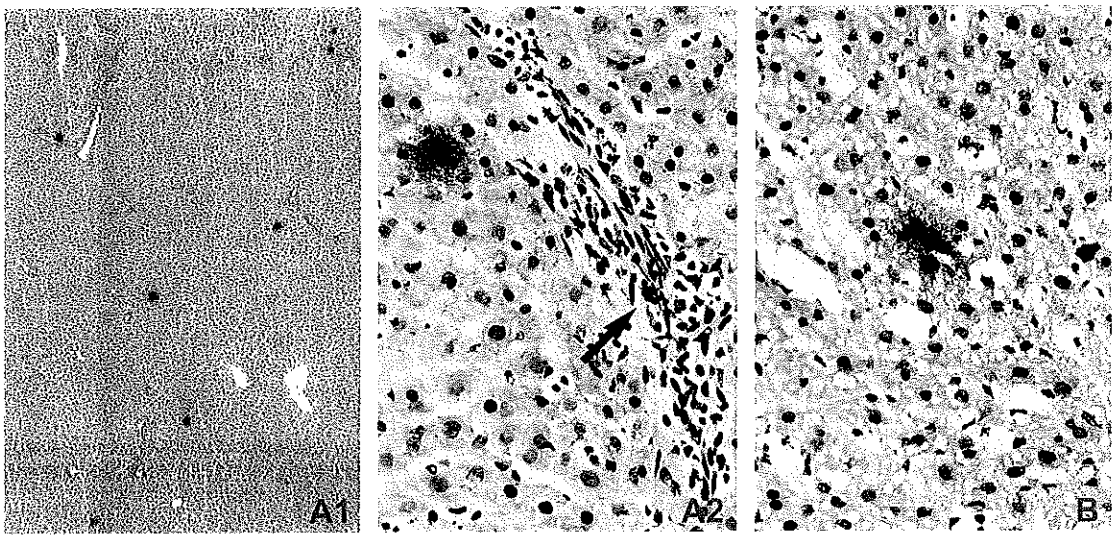

FIGURE 4 X-gal staining of liver samples 1 day after treatment with large sized (500–800 nm) and negatively charged Len DNA. Rats were injected with liposome formulations containing β-galactosidase expression vector. One day after the injection the rats were sacrificed and their livers were removed. Histochemical staining of β-galactosidase was performed as described in Materials and Methods section. A: Liver sections were taken from an intact rat treated with 0.1 mg of Len DNA into the bile duct. Positive cells were hepatocytes and probably bile ductule cells (A2: arrow). (A1: ×60, A2: ×400.) B: Liver sections were taken from a rat treated with 2.5 mg of Len DNA into the portal vein immediately after 70% PHX. Positive cell was morphologically defined as Kupffer cell. (×400). (SEE COLOR PLATE VI.)

B. Intrabile Duct Injection in Rats

Gene Transfer into the Whole Liver

Figure 5A shows the effect of the DNA dose on gene expression. Gene expression was already maximal after injection of 0.1 mg of DNA/rat and 1 day after bile duct injection, the luciferase activity in the liver reached 11.0 ± 0.9 pg/mg of protein. This level of expression represents a 10-fold increase compared to the level obtained after a similar amount of naked DNA injection (Fig. 5B) and was a 100-fold higher than the level obtained in rats injected with 2.5 mg of Len DNA into the portal vein. A similar level of luciferase activity was obtained even under slow injection condition: a total volume of 1.2 ml (1 ml of 0.1 mg Len DNA plus 0.2 ml saline) was infused during 60 min at a flow rate of 0.02 ml/min (14.6 ± 3.4 pg/mg of protein, $n = 3$). Smaller-sized liposomes were also used as DNA-carriers, but injection of these complexes resulted in lower levels of the luciferase protein production in the liver (Fig. 5B).

The tissue distribution of gene expression was assessed 1 day after Len DNA injection into the bile duct, and as illustrated in Fig. 6, the bile duct injection was liver-specific, transgene expression being detectable only in the liver and not in other organs (relative light unit in all other organs was under the detection limit). The majority of liver cells transfected by using the bile duct route of administration was identified as hepatocytes, which were relative large polygonal cells and parts of

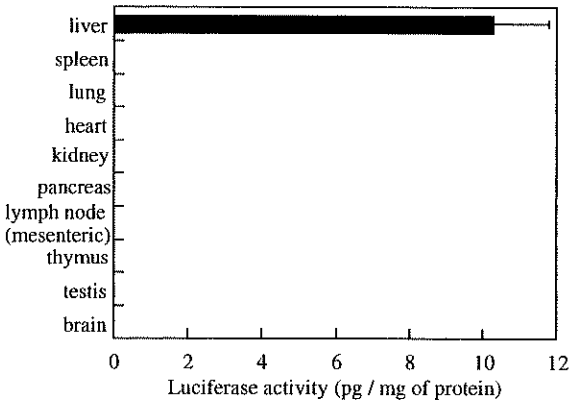

FIGURE 6 Luciferase activity in various tissues 1 day after large sized (500–800 nm) and negatively charged Len DNA injection into the bile duct in intact rats. Len DNA (pCI-luc-PRE) (0.1 mg) was injected through the bile duct in three animals. One day after Len DNA injection, animals were sacrificed and several organs were removed. The specific activity of luciferase in each sample was calculated as described in Fig. 1. The results are expressed as mean ± SE.

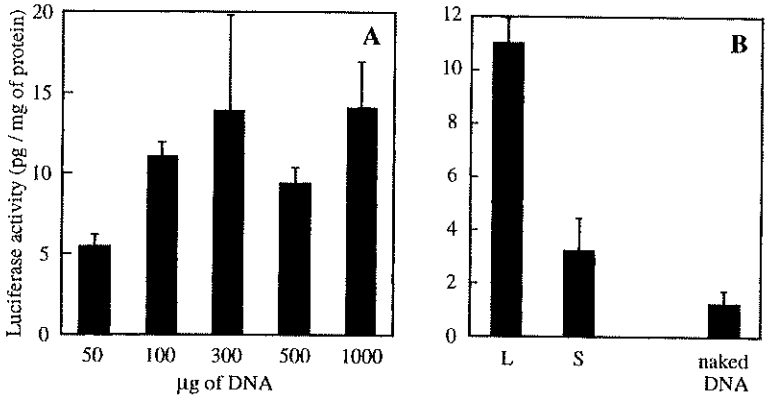

FIGURE 5 Comparison of different transfection parameters in rat liver. Len DNA (pCI-luc-PRE) was injected into the bile duct in groups of 3–6 intact animals. The specific activity of luciferase in each samples was measured 1 day after Len DNA injection as described in Fig. 1. The results are expressed as mean ± SE. A. Dose-response. Several doses of plasmid DNA were encapsulated in large sized (500–800 nm) and negatively charged liposomes. B. Size of liposomes. Plasmid DNA (0.1 mg) was encapsulated in smaller (170–200 nm) or larger (500–800 nm)-sized negatively charged liposomes. The same amount of naked DNA (pCI-luc-PRE) was also used as a control. L: larger-sized liposomes, S: smaller-sized liposomes.

single-cell-thick plates (Fig. 4A). Although small cells in the portal tract, probably bile ductule cells, were also stained in these rats, no positive cells were observed in larger bile ducts. The number of positive cells seemed higher than that the one observed after intraportal vein injection of Len DNA: 4–5 cells in 10 mm^2 of liver section were evidenced.

In biological assays, no increase in γ-GTP and total bilirubin was observed 1 day after Len DNA injection into the bile duct. Although ALT and AST levels were slightly increased (ALT: 113 ± 9.9; AST: 252 ± 18 IU/l, $n = 6$), these levels were comparable to those obtained in rats receiving the canulation only (without any injections), which was remained in the bile duct (ALT: 102 ± 22; AST: 302 ± 39 IU/l, $n = 3$).

As previously assessed after portal vein injection, the time course of transgene expression was studied after bile duct injection of 0.1 mg/rat of Len DNA (pCI-luc-PRE). The highest luciferase activity was observed 1 day after Len DNA injection but gene expression was still detectable 3 and 6 days after one single injection, as illustrated in Fig. 7.

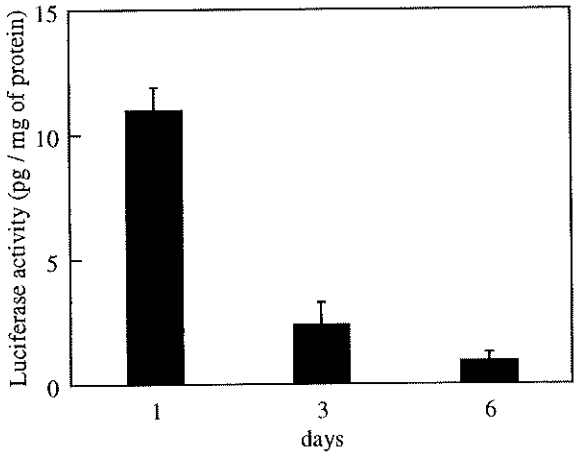

FIGURE 7 Time course of luciferase activity in the liver following 0.1 mg of large sized (500–800 nm) and negatively charged Len DNA (pCI-luc-PRE) injection into the bile duct in groups of 6 intact rats. Animals were sacrificed 1, 3 and 6 days after Len DNA injection and their livers were removed. The specific activity of luciferase in each sample was calculated as described in Fig. 1.

Effects of Partial Hepatectomy and Colchicine Treatment on Transgene Expression

A 70% PHX performed immediately before 0.1 mg Len DNA (pCI-luc-PRE) injection in the bile duct significantly increased the luciferase activity 1 day after surgery (36.8 ± 2.0 pg/mg of protein, $n = 3$). This increased transgene expression did not result from the higher DNA dose per g of remaining liver, since a three times higher dose (0.3 mg DNA) did not increase the 1 day gene expression in rats without hepatectomy (this DNA dose per liver volume being equivalent to that in rats injected with 0.1 mg of Len DNA into the remnant liver after 70% PHX). PHX, however, failed to prolong the gene expression, the luciferase activity dropping to 0.22 pg/mg of protein ($n = 2$), 6 days after surgery and Len DNA injection.

We next examined the effect of colchicine and whether a PHX performed several days after Len DNA injection into the bile duct could modify the transgene expression. As shown in Table II, PHX 6 days after injections of 0.1 mg of Len DNA (pCI-luc-PRE) did not increase but maintained the transgene expression (Group 1). Consequently, the luciferase activity 7 days after Len DNA injection was significantly higher than in rats which had undergone sham operation instead of PHX (Group 2). Animals treated with colchicine (Group 3) displayed higher luciferase activity as

TABLE II Effects of PHX 6 days after Len DNA injection and colchicine on luciferase expression in rats

Group	PHX	Colchicine	Luciferase activity (pg/mg of protein)	
			Day 6	Day 7
1	+	−	0.9 ± 0.08	1.1 ± 0.09[*,†]
2	−	−	N.D.	0.3 ± 0.1[‡]
3	+	+	4.8 ± 1.4	5.3 ± 2.1[¶,§]
4	−	+	N.D.	5.6 ± 1.8

The results were expressed as mean ± SE (groups 1 and 2, $n = 4$; groups 3 and 4, $n = 6$).
[*] $P < 0.05$ versus group 2 (day 7); [‡] $P < 0.05$ versus group 4 (day 7); [¶]N.S. versus group 4 (day 7), by Mann–Whitney U test.
[†]N.S. versus group 1 (day 6); [§]N.S. versus group 3 (day 6), by paired t test.
N.D.: not determined.

TABLE III Luciferase expression in the liver following Len DNA injection in pigs

Pig no.	Sex	BW (kg)	Liposome size (nm)	DNA amount (mg)	Injection site	Luciferase activity (pg/mg of protein)			
						Day 1	Day 2	Day 3	Day 6
1726	Male	21	500–800	15	Portal vein	N.D.	0*	N.D.	0
2301†	Male	13	170–200	70	Portal vein	0.52	0	N.D.	0
2282†	Male	12	500–800	133	Portal vein	0.20	0.03	N.D.	0
2790	Male	10	500–800	6.5	Bile duct	0.45	N.D.	0.14	0.10
2789	Female	11.5	500–800	7.5	Bile duct	0.11	N.D.	0	0
2812	Male	14	500–800	15	Bile duct	0.22	N.D.	0.13	0.14
2811	Female	16	500–800	15	Bile duct	0.13	N.D.	0.10	0

*0 means below the detection limit; †OLT was performed before Len DNA injection; N.D.: not determined.

compared to that in colchicine-untreated rats (Group 1) 6 days after Len DNA injection. This value persisted until the following day at least, and was not influenced by PHX (Groups 3 and 4).

C. Transgene Expression in the Pig Livers

The luciferase activity was detectable 1 day after Len DNA injection into either the portal vein or the bile duct, but was significantly lower than that in rats (Table III). When portal vein was used as administration route, transgene expression was only detected after OLT. The time course of transgene expression was similar to that in rats and the expression, although low, lasted longer (up to day 6) when the intrabile duct route was used. In addition, lower amounts of DNA were needed to achieve the same level of luciferase activity after injection into the bile duct than in the portal vein, even in intact pigs.

DISCUSSION

In the present study, we used negatively charged conventional large liposomes as DNA carrier, since these vectors have some advantages (Baru *et al.*, 1998; Lee and Huang, 1997). Since the plasmid DNA, a polyanionic molecule, is carried by these liposomes through encapsulation, the size and conformation of DNA are theoretically unlimited. Most neutral or anionic lipids that exist in nature are biodegradable, non-toxic and non-immunogenic, while cationic lipids were found to be toxic (Raz *et al.*, 1994). The use of these lipids rather than cationic lipids, which do not exist in nature, may be potentially more compatible *in vivo*. Large scale of liposomes can be easily produced. More importantly, some modifications which may improve gene transfer efficiency are straight forward. Several different macromolecules can easily be co-encapsulated within the same liposomes, including, for example, peptides that disrupt lysosome function or increase the delivery of the DNA from the cytoplasm to the nucleus and proteins which increase DNA stability. Indeed, Baru *et al.* (1998) have shown that a lysosome-disrupting peptide was co-encapsulated in similar type of liposomes in this study without any change of DNA encapsulation efficiency. Some molecules which may facilitate the cellular uptake of Len DNA also can be bound to the liposomal membrane.

By using the intraportal vein route, low gene transfection efficiency and transient gene expression were obtained in rats despite the high amount of DNA injected. This low efficiency could be the result of the use of large size of liposomes (500–800 nm) which limit access to hepatocytes and are rapidly taken up and degrade in the reticuloendothelial system, especially in the liver (Kupffer cells) and the spleen (Baru *et al.*, 1995; Wu and Zern, 1996). In our experimental setting, luciferase gene expression was mainly obtained through transfection of Kupffer cells as demonstrated by X-Gal

staining after Len DNA injection into the portal vein. In theory, if the size of liposomes is reduced, these may pass through the fenestrations of hepatic sinusoids after intravascular injection of liposomes. In fact, Alino *et al.* (1993) and Hara *et al.* (1987) have shown that small liposomes (less than 130 nm) could be transferred into hepatocytes, while large liposomes are trapped by Kupffer cells. In our model, however, a reduction in the size of liposomes, to 170–200 nm, did not increase the level of gene expression. This might be because our smaller-sized liposomes were still large to access directly to hepatocytes. In fact, it was difficult to reduce the size to less than 100 nm (almost same size as plasmid DNA), since encapsulated DNA was loosed and damaged due to high pressure used for the filtration of liposomes (data not shown). Composition of liposomes may also contribute to this result, since liposomes composed of EPC, cholesterol and phosphatidylserine with a size of 200–400 nm were reported to distribute in about equal amounts to Kupffer cells and hepatocytes after intravenous injection, whereas liposomes composed of EPC, cholesterol and PG were only taken up by the Kupffer cells (Daemen *et al.*, 1997).

In comparison with the intraportal vein route, bile duct injection resulted in higher and longer-lasting luciferase expression following the use of even a 25-fold lower dose of Len DNA. In this setting, transgene-expressing cells were mainly hepatocytes, indicating that Len DNA could reach the hepatocytes. Since these cells directly interface with bile canaliculi, DNA molecules might easily be able to access them, as previously discussed by Takehara *et al.* (1995), who reported successful delivery of DNA into hepatocytes by using intrabile duct injections of DNA–lipofectin complexes. Theoretically, the access of molecules to the hepatocytes after intrabile duct injection is either through bile canaliculi or via routes outside the biliary tract. The latter possibility is arisen from the investigation by Peterson and Fujimoto (1975). However, this assumption is not likely to be involved in the present study, since, once leaving the biliary space, large liposomes have no way to contact with hepatocytes. Even if the size or conformation of liposomes were modified during this process, they would still need to escape being taken up by reticuloendothelial system. Thus, although direct evidence has not been shown in this study, it is conceivable that our large liposomes entered hepatocytes through bile canaliculi. Since liposomes are not soluble and the size is large, they may remain in the biliary tract even whether the injected volume exceeded the volume of biliary tree. As a result, Len DNA delivered by this route could thus avoid rapid scavenging by Kupffer cells and the large size liposomes (500–800 nm) had no difficulty reaching the hepatocytes. On the contrary, large size vectors may be advantageous by this route, and we found that, by using smaller-sized liposomes, the level of gene expression was decreased. Moreover, the bile duct route allowed liver-specific gene transfer and these results suggest that *in vivo*, the bile duct might be an ideal administration route for large multilamellar liposome-mediated gene transfer into the liver.

In this study, we infused saline after liposome injection. The goal of this infusion was to move liposomes toward bile canaliculi and to avoid the back flow of liposomes toward the common bile duct during its occlusion. It is not clear whether this goal was achieved, but, in our preliminary experiment, the levels of gene expression after temporary ligation of the bile duct only were lower than those in the current protocol and varied greatly among the animals despite great improvement of gene expression as compared with those after Len DNA injection into the portal vein (data not shown). Further systematic study assessing the influence of volume and flow rate on gene expression, as well as potential damages of the transport of bile acids, cholesterol, phospholipids and dyes, although the levels of ALT, AST, γ-GTP and total bilirubin were not affected in this study, are ongoing.

A single injection of colchicine, which is known to disrupt microtubules, contributed to the persistence of a high level of luciferase activity for at least 7 days. Similar results have been reported in the study of gene transfer via the tail vein of rats by asialoglycoprotein receptor-mediated endocytosis (Chowdhury

et al., 1996). Since a microtubular network is required for the translocation of endosomes to lysosomes (Matteoni and Kreis, 1987), the authors postulated that transient microtubular disruption results in its failure and endosomal vesicles modified during the period required for regeneration of the disrupted microtubules circumvent subsequent association with microtubules or functional motor proteins. Our results suggest that intracellular transport after internalization of Len DNA injected into the bile duct depends on the microtubules.

In clinical situation, local and transient gene transfer into the liver, following PHX and OLT have important applications. For instance, local immunomodulation against micrometastases of a variety of primary or secondary cancers or allograft rejection could be the target for gene therapy. It therefore seemed important to investigate the effects of such manipulations on foreign gene expression. Previous reports (Wu *et al.*, 1989; Crespo *et al.*, 1996; Hirano *et al.*, 1998) have shown that PHX resulted in sustained gene expression, but enhanced expression after gene transfer was not observed, as it was in our study. Similar increased gene expression was found in our experiments when Len DNA was injected immediately after OLT or ischemia. This increased gene expression is not related to a regenerative process, since OLT and liver ischemia have no or weak ability to induce liver regeneration. The fact that PHX performed 6 days after Len DNA injection failed to increase gene expression in colchicine-treated rats suggests that some event occurred before access of DNA to the nucleus. Although the mechanism of enhancement is not clear, the increase in hydrostatic pressure of the portal vein or in the biliary tree may be involved. In fact, all surgeries examined in this study have been reported to increase the portal vein pressure (Sato *et al.*, 1997; Jakab *et al.*, 1996; Suzuki *et al.*, 1998). The pressure increase after PHX depends on the amount of resection and lasts through liver regenerative process (Sato *et al.*, 1997), while in liver ischemia (20 min), normal portal vein pressure might be quickly restored following reperfusion. Zhang *et al.* (1997) have shown the correlation between parenchymal pressure and transgene expression after naked DNA injection into the portal vein and have speculated that increased pressure may enhance cellular internalization processes. In most of previous reports, DNA was injected before PHX. Only Hirano *et al.* (1998) performed 70% PHX 1 day before *in vivo* transduction, but these authors used the hemagglutinating virus of Japan-liposome which is a vector with different mechanism of cellular uptake. Endocytic process may be enhanced by PHX, ischemia or OLT.

A PHX immediately before Len DNA injection into the bile duct did not contribute to the persistent expression of transfected gene, although pretreatment of colchicine did. These results were inconsistent with previous reports (Chowdhury *et al.*, 1993; 1996; Wilson *et al.*, 1992; Bommineni *et al.*, 1994), in which a marked decrease in microtubular structure was seen early after PHX, leading to similar effects as seen with colchicine injection after receptor-mediated gene transfer. Fukuyama *et al.* (1988), however, reported the converse, in which microtubules were increased after PHX. Despite the timing of PHX (before or after DNA injection), endocytosis (receptor-mediated or not) and the direction of intracellular transport ((−) end- or (+) end-directed) were different, so it is likely that other mechanisms are involved in this issue. The effect of PHX performed 6 days after Len DNA injection could not be explained by the increased cellular uptake discussed above or by the microtubular disruption, since, at this time point, DNA might already have entered the nucleus. Simultaneous event taking place in the nucleus may be responsible for this maintenance of gene expression.

Successful non-viral gene transfer techniques have been reported in small animals, but there are few such reports in large animal models. Our results in pigs indicate that liposomes might work as DNA-carriers in large mammals. Similarly, as in our rat experiments, preliminary success was obtained by using the bile duct route, with a low amount of Len DNA, to obtain luciferase activity in the liver of intact pigs. The level of gene expression, however,

was lower than in rat experiments. The cause of this less efficiency was not clear, thereby leaving room for further investigation and optimization.

In summary, large-sized liposomes successfully used as gene transfer vectors into hepatocytes through the bile duct, achieving liver-specific gene expression. These results indicate that intrabile duct route might render large non-viral vectors applicable to gene transfer into the hepatocytes. However, the level of gene expression was still low by using conventional Len DNA only, and, therefore, further optimization is required. The transfection efficiency of liposome-mediated gene transfer into the liver can be increased by several surgical manipulations such as PHX, ischemia or OLT performed before DNA injection.

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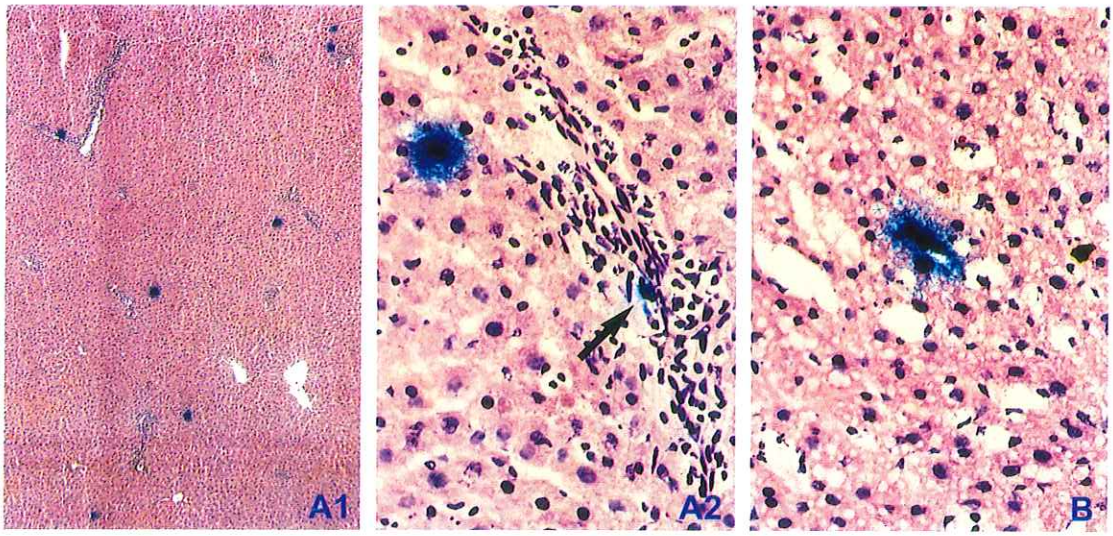

JOURNAL OF DRUG TARGETING: VOLUME 8 COLOUR PLATE VI.
See M. Otsuka *et al.*, Figure 4.