Disposition Characteristics of Plasmid DNA in the Single-pass Rat Liver Perfusion System

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Received August 14, 1995; accepted January 2, 1996

Purpose. To define the hepatic uptake mechanism of a plasmid DNA, we quantitated the uptake of pCAT (plasmid DNA encoding chloramphenicol acetyltransferase reporter gene fused to simian virus 40 promoter), a model plasmid, after a single pass through the perfused rat liver using albumin- and erythrocyte-free Krebs-Ringer bicarbonate buffer (pH 7.4).
Methods. [^{32}P]pCAT was introduced momentarily into this system from the portal vein as a bolus input or constant infusion mode, and the outflow patterns and hepatic uptake were evaluated using statistical moment analysis.
Results. The venous outflow samples had electrophoretic bands similar to that of the standard pCAT, suggesting that the plasmid is fairly stable in the perfusate during liver perfusion. In bolus experiments, pCAT was largely taken up by the liver and the uptake was decreased with increase in injected dose. Statistical moment analysis against outflow patterns demonstrated that the apparent volume of distribution of pCAT was greater than that of human serum albumin, indicating a significant reversible interaction with the tissues. The results of collagenase perfusion experiments suggest that the hepatic accumulation of pCAT occurred preferentially in the nonparenchymal cells (NPC). The amount of total recovery in the liver decreased substantially by preceding administration of polyinosinic acid, dextran sulfate, succinylated bovine serum albumin, but not by polycytidylic acid. This suggests that pCAT is taken up by the liver via scavenger receptors for polyanions on the NPC. In constant infusion experiments, the presence of 2,4-dinitrophenol and NH_4Cl caused a significant increase in the outflow concentration of [^{32}P]pCAT and decrease by half in the total hepatic recovery than that of plasmid DNA administered alone, suggesting that plasmid DNA may undergo internalization by the NPC.
Conclusions. The liver plays an important role in the elimination of plasmid DNA and a successful delivery system will be required to avoid its recognition by the scavenger receptors on the liver NPC.

KEY WORDS: plasmid DNA; liver perfusion; pharmacokinetics; gene therapy.

INTRODUCTION

The use of non-viral vectors in combination with specific macromolecular ligands (1) and liposomes (2) is a safe and attractive approach for *in vivo* gene therapy. As *in vivo* gene expression is greatly influenced by the stability, disposition and cellular uptake mechanism of the plasmid, clinical applications will require detailed knowledge of pharmacokintetics of the gene and its product. Hence, we need to examine the *in vivo* pharmacokinetic properties of plasmid DNA in detail since the rational delivery systems should be designed based on information of the disposition characteristics of naked plasmid DNA.

We have been studying systematically the pharmacokinetic properties of macromolecules in relation to their physicochemical properties such as molecular weight and electric charge. On the basis of these findings, we have developed various kinds of carrier systems for proteins and have demonstrated their usefulness for therapeutic uses (3).

Previously, we studied the stability and pharmacokinetics of pCAT (4) and demonstrated that the liver plays an important role for the elimination of plasmid DNA administered intravenously. Hence, in the present study, we examined the hepatic disposition characteristics of plasmid DNA using the single pass rat liver perfusion system, aiming at obtaining detailed information on the hepatic uptake mechanism of the plasmid.

MATERIALS AND METHODS

Materials

Polyinosinic acid (poly [I], mw 170–1000 kDa), polycytidylic acid (poly [C], mw 170–1900 kDa), and 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulfonic acid (SITS, mw 498.5 Da) and dextran sulfate (DS, mw 150 kDa) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A). Clear-sol I and 2, 4-dinitrophenol (DNP, mw 184.1 Da) were purchased from Nacalai Tesque (Tokyo, Japan). [α-^{32}P]dCTP (800 Ci/mmol) was obtained from NEN Research Products (Boston, MA, U.S.A.). pCAT-Control Vector and Soluene-350 were purchased from Promega (Madison, WI, U.S.A) and Packard (Netherlands), respectively. All other chemicals were obtained commercially as reagent-grade products. Succinylated bovine serum albumin (Suc-BSA, mw 70 kDa) was synthesized and purified as described previously (3).

Preparation of Plasmid DNA

Plasmid DNA was amplified in the HB101 strain of *Escherichia coli*, extracted by the alkaline lysis technique, purified by the precipitation with polyethylene glycol, and diluted with sterilized phosphate-buffered saline. Purity was confirmed by 1% of agarose gel electrophoresis followed by ethidium bromide staining and the DNA concentration was measured by UV absorption at 260 nm. The plasmid was labeled with [α-^{32}P]dCTP by nick translation.

Liver Perfusion Experiment

Male Wistar rats (180–200 g) were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan) and liver was perfused *in situ* as described by Nishida *et al.* (5). The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg), the abdomen and chest were opened, and the portal vein and the inferior vena cava were cannulated with a polyethylene tubing (PE-160). Freshly prepared and filtered (0.2 μm) albumin- and erythrocyte-free Krebs-Ringer bicarbonate buffer (pH 7.4), supplemented with 10 mM glucose was oxygenated with 95% O_2: 5% CO_2, and delivered to the portal vein catheter via a peristaltic pump at a constant flow rate of 13 ml/min. The temperature

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of the perfusion cabinet and perfusion medium was thermostatically controlled at 37°C. The bile duct was cannulated with polyethylene tubing (PE-10) and bile was collected into the preweighed tubes at 10 min interval for 1 hour. An initial stabilization period of 30 min was allowed before introducing [^{32}P]pCAT dissolved in the perfusion medium into the portal vein using a six-rotary valve injector. Venous outflow samples were collected up to 1 min into the preweighed tubes at 0.5 to 3 sec intervals. The sample volumes were estimated from the gain in weight in the tube assuming the density of the outflow perfusate to be 1.0. The sampling time was calculated from each sample volume, assuming a constant flow rate. After the perfusion experiment, the whole liver was excised, weighed and homogenized.

For determining the intracellular localization of pCAT, perfusion of liver perfusion buffer containing 5 mM $CaCl_2$ and 0.05% (w/v) collagenase was started 15 min after DNA administration (1.33 μg/liver) and parenchymal cells (PC) and non-parenchymal cells (NPC) were separated by centrifugation as described previously (5). The number of cells and viability were determined by the trypan blue exclusion method.

Analytical Methods

The radioactivity of the effluent perfusate and bile samples was measured using a liquid scintillation counter (LSA-500, Beckman, Tokyo, Japan) after addition of Clear-sol I. The radioactivity in the homogenized liver or cell suspensions was measured in the same manner after dissolution with Soluene-350 through incubation overnight at 45°C and neutralization with 2N HCl. To check the stability of [^{32}P] label on plasmid DNA, the outflow samples were collected from the constant infusion experiment, applied to 1% agarose gel, and electrophoreshed. The gel was dried and autoradiographed (BAS 2000, Fuji Photo Co. Ltd., Japan).

Pharmacokinetic Analysis of Outflow Patterns

We have previously described the detailed theoretical background of moment analysis for local injection (6) and its application to analyze the hepatic disposition of macromolecular carriers (7). In this paper, outflow patterns were also analyzed by using the statistical moment theory. The statistical moment parameters for the outflow pattern are defined as follows:

$$AUC = \int_0^\infty Cdt \quad (1)$$

$$\bar{t} = \int_0^\infty tCdt/AUC \quad (2)$$

where t is the time and C is the concentration of compounds normalized with respect to the percentage of injection dose per ml. AUC and $\bar{t}$ denote the area under the concentration-time curve and mean transit time of the drug through the liver, respectively. The moments defined by Eqs. (1) and (2) can be calculated by numerical integration using a linear trapezoidal formula and extrapolation to infinite time based on a monoexponential equation (8). The $\bar{t}$ values were corrected for the lag time of the catheter.

The hepatic disposition parameters of plasmid DNA, representing reversible and irreversible processes, were calculated using the following equations:

$$F = AUC.Q/100 \quad (3)$$

$$V = \bar{t}/AUC.100 \quad (4)$$

$$E = 1 - F \quad (5)$$

$$t_{corr} = \bar{t}/F \quad (6)$$

$$CL_{int} = Vk_{el} \quad (7)$$

where F is the recovery ratio; V is the apparent distribution volume, reflecting reversible interaction; t_{corr} is the corrected mean transit time, E is the extraction ratio, K_{el} is the first-order irreversible elimination rate constant; CL_{int} is the intrinsic clearance, and Q is the perfusion rate. These parameters can be divided into three groups, i.e., parameters representing reversible (V and t_{corr}), irreversible (E, F, k_{el}) processes, and both (CL_{int}).

Inhibitory Effect of Polyanions on Hepatic Uptake of Plasmid DNA

[^{32}P]pCAT (1.33 μg/liver) and unlabeled polyanion (DS, poly [I], suc-BSA, poly [C]) or SITS (26.6 μg/liver) were co-injected in the single-pass liver perfusion model. At 30 min after DNA administration, the liver was harvested and subjected to radioactivity assay. The constant infusion experiment of [^{32}P]pCAT was performed for 60 min in the absence (control experiment) and presence of 100 μM DNP and 20 mM NH_4Cl, and the radioactivity in the liver was compared.

RESULTS

Stability of Plasmid DNA in the Outflow Samples

Agarose gel electrophoresis was employed to check the stability of [^{32}P]pCAT in the liver perfusion buffer after 60 min incubation at 37°C. All samples had similar bands, suggesting that the plasmid DNA was fairly stable in the perfusate (data not shown). The stability of [^{32}P]pCAT during the constant infusion experiment was also checked by agarose gel electrophoresis and the result is shown in Figure 1. The electrophoretic

Electrophoresis Pattern of [^{32}P] pCAT Appearing in Venous Outflow

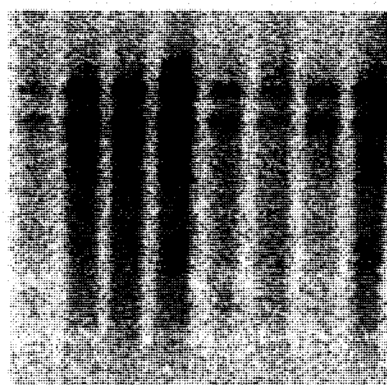

Fig. 1. Gel electrophoretic patterns of [^{32}P]pCAT appearing in venous outflow perfusate. 5, 10, 20, 30, 40, 50, and 60 represent sampling time in minutes at which venous outflow perfusate was collected for gel electrophoresis. OC, open circular; L, linear; Std, standard.

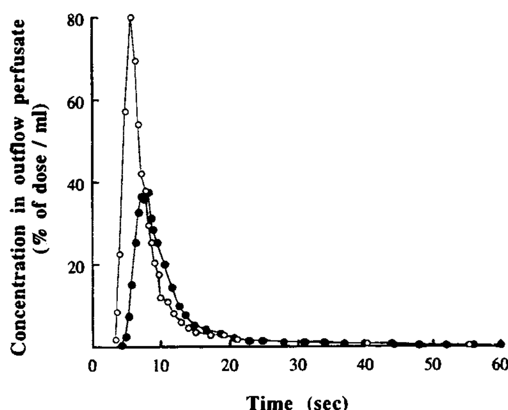

Fig. 2. Typical venous outflow patterns of [^{32}P]pCAT in the single-pass rat liver perfusion system. Doses: ○, 13.3 and ●, 1.33 μg/liver.

patterns of these samples were similar to that of the standard. Moreover, polyacrylamide gel electrophoresis were performed to check the low molecular weight-degradation products of [^{32}P]pCAT, but no degradation could be observed (data not shown).

Outflow Patterns After Single-pass Liver Perfusion

Figure 2 illustrates typical outflow concentration-time curves of [^{32}P]pCAT after bolus administration at doses of 1.33 and 13.3 μg/liver. The 13.3 μg/liver dose sample gave an extremely high peak concentration of 80% of dose/ml, while 1.33 μg/liver dose sample gave a low peak concentration of about 40% of dose/ml.

Pharmacokinetic Analysis of Outflow Patterns

Table 1 summarizes the pharmacokinetic parameters derived from concentration-time curves. The results for ^{51}Cr-RBC and ^{131}I-HSA were referred to as vascular reference substance from our previous report (5). The distribution volumes for ^{51}Cr-RBC and ^{131}I-HSA correspond to the volume of the sinusoidal space (0.209 ml/g liver) and sinusoidal space plus the space of Disse (0.252 ml/g liver), respectively. The plasmid DNA samples showed a large distribution volume (0.399–0.688 ml/g), indicating that there is an interaction between plasmid DNA and the liver. These parameters showed dose dependency as the value of V decreased from 0.598 to 0.314 ml/g liver as the injection dose increased from 1.33 to 13.3 μg/liver. The dose dependent uptake mechanism of plasmid DNA in the liver was also supported by the E value which decreased from 45.6% to 20.1%. For 1.33 μg DNA/liver sample, the actual liver accumulation at 60 min after injection was 36.57 ± 3.28 (% dose/liver), which is in good agreement with the calculated E value. In the case of 4°C perfusion, the tissue uptake was 42.50 ± 2.83 (% dose/liver), which was not significantly different from the 37°C data. Moreover, plasmid DNA was undetectable in bile during the entire course of the study.

Cellular Localization of Plasmid DNA

Fig. 3 shows the results of collagenase perfusion experiments of 1.33 μg/liver dose samples. The recovery of PC and NPC was 0.73 and 2.85% of the dose/10^8 cells, respectively. The theoretical numbers of PC and NPC of rat liver are 1.25 × 10^8 and 0.65 × 10^8 cells/g liver, respectively (9). On the basis of these theoretical values, the recovery of PC and NPC was calculated as 0.91 and 1.85% of the dose/g liver, respectively. These findings suggest that plasmid DNA was preferentially taken up by NPC.

Inhibitory Effects of Polyanions on Hepatic Uptake of Plasmid DNA

Coadministration of DS, poly [I], and suc-BSA caused a substantial decrease in the amount of total recovery of [^{32}P]pCAT in the liver (Figure 4). Poly[I] was the most effective among the polyanions. However, coadministration of poly[C]

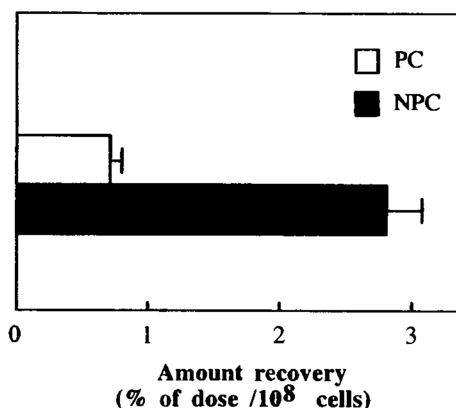

Fig. 3. Hepatic cellular localization of radioactivity [^{32}P]pCAT in the single-pass rat liver collagenase perfusion. The results are expressed as the mean ± S.D. (standard deviation) of three rats.

Table 1. Moments and Disposition Parameters for Plasmid DNA and Reference Compounds in Single-pass Rat Liver Perfusion System

		Moment parameters		Disposition parameters				
Samples	Dose (μg/liver)	AUC (% of dose.sec/ml)	$\bar{t}$ (sec)	V (ml/g)	t cor (min)	E (%)	kel (min^{-1})	Clint (ml/min/g)
*^{32}P-pCAT	1.33	251.5 ± 16.92	12.61 ± 1.45	0.598 ± 0.09	0.313 ± 0.05	45.56 ± 0.31	2.168 ± 0.128	1.29 ± 0.12
	13.3	365.2 ± 13.50	10.31 ± 1.03	0.314 ± 0.08	0.179 ± 0.03	20.12 ± 0.75	1.171 ± 0.09	0.354 ± 0.10
#^{51}Cr-RBC	—	471.3	8.89	0.209	0.148	0	—	0
#^{131}I-HSA	—	485.9	9.33	0.252	0.156	0	—	0

*Results are expressed as the mean ± S.D. (standard deviation) of three experiments.
#Published results (5).

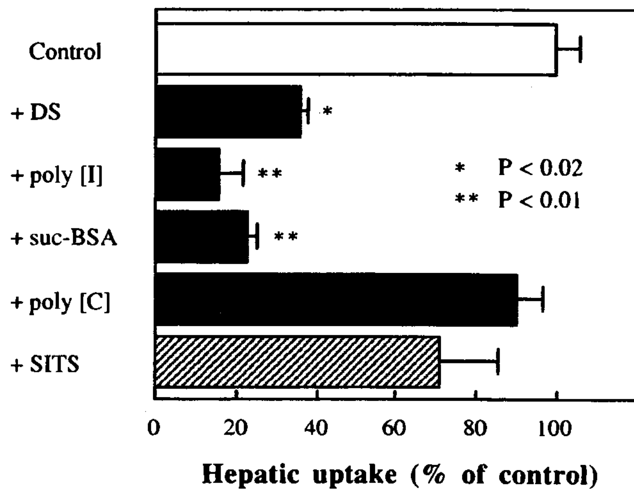

Fig. 4. Inhibitory effects of polyanions and SITS on hepatic uptake of plasmid DNA in the single-pass liver perfusion. [^{32}P]pCAT and inhibitory compounds were coadministered to the portal vein and after 30 min, the liver was harvested and subjected to radioactivity assay. The results are expressed the mean ± S.D. (standard deviation) of three rats.

and SITS or 20-min pretreatment of SITS had no effect on the hepatic uptake of pCAT.

Figure 5 shows the typical outflow patterns and the total amount of radioactivity in the liver of [^{32}P]pCAT administered in the presence of DNP and NH_4Cl. The outflow concentration of [^{32}P]pCAT was higher and the total hepatic accumulation was much lower than that of pCAT administered alone.

DISCUSSION

We previously demonstrated that plasmid DNA was cleared rapidly from the blood circulation by the liver, and DNA remaining in the circulation is degraded by plasma DNase and/or tissue nucleases (4). Emlen and Mannik (10) also observed rapid hepatic uptake of large double stranded DNA from the blood circulation and degradation by the serum and tissue nucleases. Due to such rapid clearance and breakdown, intact DNA can hardly persist in the circulation long enough to come in free contact with the liver and other organs.

The use of a buffer containing erythrocytes and serum albumin is necessary for maintaining better physiological functions of the liver during perfusion experiments. It is also better to use these blood components to correlate the results of liver perfusion experiments with *in vivo* results. However, to avoid possible contamination of nucleases from erythrocytes and albumin, liver perfusion was carried out using a perfusion buffer containing no blood components.

Gel electrophoretic patterns of albumin- and erythrocyte-free Krebs-Ringer bicarbonate buffer (pH 7.4) containing [^{32}P]pCAT after 37°C incubation or after constant infusion suggests that plasmid DNA is fairly stable in the perfusate during liver perfusion experiments as these samples had bands similar to that of the standard pCAT. The apparent discrepancy between rapid *in vivo* degradation of plasmid DNA (4) and the absence of DNA breakdown in the perfused liver may be due to the absence of serum nucleases in the perfusate.

Plasmid DNA was largely taken up by the liver and resulted in a large volume of distribution (Table 1). More than 40% of 1.33 μg/liver dose sample was trapped by the liver during single passage. The V value for plasmid DNA was greater than that for HSA (vascular reference substance) and the ratio of the V values for pCAT to that of HSA was between 124 and 237%, suggesting the existence of a reversible interaction with the tissue.

Plasmid DNA was preferentially taken up by NPC. These findings suggest that the total recovery of [^{32}P]pCAT is very low (about 40% of injected dose/10^8 cells). The detection of radioactivity of ^{32}P in PC also suggests the occurrence of intercellular transport during collagenase perfusion and cell separation process. In the collagenase liver perfusion experiments, pCAT was administered at a dose of 1.33 μg/liver. Although we have not done this experiment at different doses, we speculate that the cellular localization of pCAT may be slightly influenced by the amount of DNA administered to the rat due to the possible occurrence of a saturation process at higher doses.

Coadministration of polyanions (DS, suc-BSA, poly[I]) caused a substantial decrease in the amount of total recovery in the liver. However, coadministration of poly[C] or 20-min

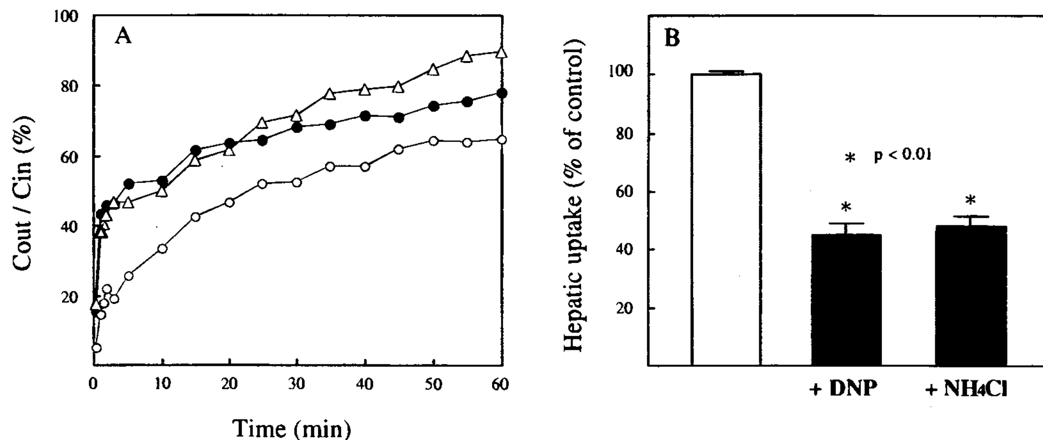

Fig. 5. Typical venous outflow patterns (A) and hepatic uptake (B) in the presence of 2,4-dinitrophenol (DNP) and NH_4Cl in the rat liver constant infusion. ○ control (0.01 μg/ml), ● +100μM DNP, △ + 20 mM NH_4 Cl.

pretreatment of SITS had no effect on the hepatic uptake of pCAT. Inhibition of hepatic uptake of pCAT does not seem to be influenced by the molecular weight or number of moles of these polyanions, but by their specific interaction with the scavenger receptors. Due to differences in molecular weight, the moles used in this study were in the following order of magnitude:- Suc-BSA > DS > poly[I] ≥ poly[C]. However, the magnitude of inhibition of hepatic uptake of pCAT by these polyanions was as follows:- poly[I] > Suc-BSA > DS. The absence of an effect on the hepatic uptake of pCAT by poly[C] is not due to the smaller moles of poly[C] used in this study, but the lack of its recognition by the scavenger receptors. Brown et al. (11) demonstrated that poly[I] completely inhibited the uptake of acetyl-low density lipoprotein (acetyl-LDL) by mouse peritoneal macrophages, whereas poly[C] showed no effect even at a concentration 20 times higher than that of poly[I]. Furthermore, the potency of poly[I] in inhibiting the binding of acetyl-LDL was independent of molecular weight between 8.3 and 198 kDa. Acton et al. (12) also reported that scavenger receptors recognize poly[I], DS, and maleylated BSA, but not poly[C]. This finding is also in good agreement with those obtained in our previous collagenase mouse liver perfusion experiments, in which pCAT was injected into the tail vein of mice (4).

Under steady state conditions in the constant infusion experiment, the elimination rate of macromolecules from the perfusate, i.e., $(C_{in}-C_{out}).Q$, corresponds to the internalization rate (13). The presence of DNP and NH_4Cl during constant infusion resulted in the increase of radioactivity in the venous out flow perfusate and decrease by half in the amount of total recovery of DNA in the liver (Figure 5). NH_4Cl, a weak base metabolic inhibitor, probably interferes with the scavenger receptor-mediated uptake of pCAT by raising the lysosomal pH. This suggests the internalization of pCAT by the scavenger receptors on the NPC. This speculation is further supported by the work of Naito et al. (14) who have reported that scavenger receptors dissociate their ligands more efficiently at an acidic condition than at a basic environment. DNP treatment probably interferes with receptor internalization by reducing cellular ATP. There was continuous production of bile during DNP treatment. Also the concentration of DNP used in this study is fairly low (100 μM) and hence even if DNP treatment reduces cellular ATP, most of the liver cells should remain viable and the ATP level should return to normal after withdrawal of DNP treatment. Backer et al. (15) have reported that the rat hepatoma cells were viable during DNP (2 mM) treatment and that ATP levels returned to >70% of the normal level after withdrawal of DNP for 1 h. These authors have suggested that the effect of DNP on the autophosphorylation of the insulin receptor is independent from its effect on receptor-mediated internalization of insulin in rat hepatoma cells. To avoid any speculation, further studies such as acid wash experiments are required to clarify whether plasmid DNA is internalized by NPC.

The findings obtained here support our previous findings obtained *in vivo* that plasmid DNA is preferentially taken up by the scavenger receptor on the liver nonparenchymal cells. Therefore, a successful delivery system will be required to avoid the recognition of plasmid DNA by the scavenger receptors on the liver nonparenchymal cells.

ACKNOWLEDGMENTS

This work was financially supported by the Ministry of Education, Science, and Culture, Japan, and the Naito Foundation, Japan.

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