ACQUIRED DISEASES

RESEARCH ARTICLE

Stabilized plasmid-lipid particles for systemic gene therapy

P Tam^{1*}, M Monck^{1*}, D Lee¹, O Ludkovski¹, EC Leng¹, K Clow¹, H Stark², P Scherrer³,
RW Graham¹ and PR Cullis^{1,3}

¹Inex Pharmaceuticals Corporation, Burnaby, Canada; ²Institute for Molekularbiologie und Tumorforschung, Marburg, Germany; and ³Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada

The structure of 'stabilized plasmid-lipid particles' (SPLP) and their properties as systemic gene therapy vectors has been investigated. We show that SPLP can be visualized employing cryo-electron microscopy to be homogeneous particles of diameter 72 ± 5 nm consisting of a lipid bilayer surrounding a core of plasmid DNA. It is also shown that SPLP exhibit long circulation lifetimes (circulation half-life >6 h) following intravenous (i.v.) injection in a murine tumor model resulting in accumulation of up to 3% of the total injected dose and concomitant reporter gene expression at a distal (hind flank) tumor site. In contrast, i.v. injection of

naked plasmid DNA or plasmid DNA–cationic liposome complexes did not result in significant plasmid delivery to the tumor site or gene expression at that site. Furthermore, it is shown that high doses of SPLP corresponding to 175 μ g plasmid per mouse are nontoxic as assayed by monitoring serum enzyme levels, whereas i.v. injection of complexes give rise to significant toxicity at dose levels above 20 μ g plasmid per mouse. It is concluded that SPLP exhibit properties consistent with potential utility as a nontoxic systemic gene therapy vector. Gene Therapy (2000) 7, 1867–1874.

Keywords: liposomes; cancer gene therapy; intravenous gene therapy; tumour transfection

Introduction

Gene therapies for systemic diseases such as cancer or inflammatory disorders clearly require systemic vectors. However, currently available vectors for gene therapy have limited utility for systemic applications. Recombinant virus vectors, for example, are rapidly cleared from the circulation following intravenous injection, limiting potential transfection sites to 'first pass' organs such as the liver.^{1,2} Nonviral systems, such as plasmid DNA–cationic liposome complexes, are also rapidly cleared from the circulation, and the highest expression levels are again observed in first pass organs, particularly the lungs.^{3–8}

Intravenous administration of chemotherapeutic drugs encapsulated in small (diameter ≤ 100 nm), long-circulating (circulation half-life $t_{1/2} \geq 5$ h in murine models) liposomes results in preferential delivery of encapsulated drug to distal tumors due to increased vascular permeability in these regions.^{9–11} It therefore follows that intravenous injection of plasmid DNA encapsulated in small, long circulating lipid particles should give rise to preferential delivery of plasmid DNA to tumor sites. Recent work has shown that plasmid DNA can be encapsulated in small (approximately 70 nm diameter) 'stabilized plasmid-lipid particles' (SPLP) that contain one plasmid per particle.¹² These particles contain the 'fusogenic'

lipid dioleoylphosphatidylethanolamine (DOPE), low levels of cationic lipid and are stabilized in aqueous media by the presence of a poly(ethylene glycol) (PEG) coating. Here, we show that the structure of SPLP can be directly visualized employing cryo-electron microscopy to reveal homogeneous particles consisting of plasmid DNA entrapped within a bilayer lipid vesicle. Furthermore, we show that these SPLP exhibit long circulation lifetimes and no evidence of systemic toxicities following i.v. injection in a murine tumor model. Under the experimental conditions employed, approximately 3% of the total injected SPLP dose was delivered to a subcutaneous tumor site and 1.5 % of the total intact plasmid dose could be detected at the tumor site at 24 h. Significant levels of reporter gene expression were observed at the tumor site employing the SPLP system, whereas no expression was observed following i.v. injection of 'naked' plasmid DNA or plasmid DNA–cationic liposome complexes.

Results

SPLP consist of a plasmid trapped inside a bilayer lipid vesicle

Previous work has shown that plasmid DNA can be encapsulated (trapping efficiency approximately 70%) in SPLP by a detergent dialysis procedure employing octylglucopyranoside (OGP).¹² These SPLP are composed of DOPE, 5–10 mol% of the cationic lipid dioleoyldimethylammonium chloride (DODAC) and PEG attached to a ceramide anchor containing an arachidoyl acyl group

Correspondence: P Tam, Inex Pharmaceuticals Corporation, 100 – 8900 Glenlyon Parkway, Burnaby, BC V5J 5J8, Canada

*These authors contributed equally to this work.

Received 31 January 2000; accepted 18 July 2000

(PEG-CerC₂₀). SPLP can be separated from non-encapsulated plasmid by ion exchange chromatography and can then be further purified by density gradient centrifugation to remove empty vesicles produced during the dialysis procedure. On the basis of the size and plasmid-to-lipid ratio of these purified SPLP it was determined that each SPLP contained one plasmid molecule.¹²

Here, we further characterize SPLP structure employing cryo-electron microscopy. Following the procedures summarized in Materials and methods, purified SPLP were prepared from DOPE:DODAC:PEG-CerC₂₀ (83:7:10; mol:mol:mol) and pCMVluc, whereas large unilamellar vesicles (LUV) with the same lipid composition were prepared by extrusion of the hydrated lipid mixture through 100 nm pore size filters. As shown in Figure 1a, the cryo-electron micrographs clearly reveal SPLP to con-

sist of a lipid bilayer surrounding an internal structure consistent with entrapped plasmid DNA molecules. Small (diameter approximately 30 nm), empty vesicles formed during the detergent dialysis process¹³ that were not removed by density centrifugation do not exhibit such internal structure (see arrows in Figure 1a). This internal structure is also not observed in the LUV produced by extrusion (Figure 1b). It may also be noted that SPLP as detected by cryo-electron microscopy have a remarkably homogeneous size (diameter 72 ± 5 nm), in close agreement with measurements of SPLP diameter employing freeze-fracture electron microscopy (diameter 64 ± 9 nm).¹² The homogeneous size and morphology of SPLP contrasts with the irregular morphology and large size distribution of the extruded vesicles. The narrow size distribution of SPLP was also reflected by quasi-elastic light scattering (QELS) measurements (data not shown) which indicated a mean diameter of 83 ± 4 nm. Plasmid DNA-cationic liposome complexes made from DOPE:DODAC (1:1; mol:mol) LUV exhibited a large, heterogeneous size distribution as determined by QELS (diameter 220 ± 85 nm, data not shown).

SPLP exhibit extended circulation lifetimes, preferential accumulation at tumor sites, and low systemic toxicities following intravenous injection

The next set of experiments was aimed at characterizing the pharmacokinetics and biodistribution of SPLP following i.v. injection into tumor-bearing mice. SPLP were prepared with trace amounts of the lipid label, ³H-cholesteryl hexadecylether (³H-CHE) and were injected at a dose level equivalent to 100 µg plasmid DNA per mouse into C57Bl/6 mice bearing a subcutaneous Lewis lung carcinoma (approximately 200 mg) in the hind flank. The clearance of SPLP from the circulation as assayed by the lipid label (Figure 2a) corresponds to a first order process with a $t_{1/2}$ of 6.4 ± 1.1 h. Relatively low levels of uptake by the lung and liver are observed (Figure 2b and c) whereas approximately 3% of the injected SPLP dose accumulates at the tumor site over 24 h (Figure 2d). Such tumor accumulation levels are comparable with those achieved for small, long-circulating liposomes containing conventional drugs such as doxorubicin, where approximately 5% of the injected dose can be found at 24 h in larger (>0.5 g) tumors.¹⁴ In contrast to the behavior of the SPLP system, ³H-CHE-labeled plasmid DNA-cationic liposome complexes were rapidly cleared from the circulation ($t_{1/2} \ll 15$ min), appearing predominantly in the lung and liver, and less than 0.2% of the injected dose was found at the tumor site at 24 h. The biodistribution of ³H-CHE labeled SPLP and complexes 4 and 24 h following injection are summarized in Table 1. Only trace amounts were detected in kidney, heart and lymph nodes.

The levels of intact plasmid DNA in the circulation and tumor tissue following i.v. injection of naked plasmid DNA, plasmid DNA-cationic lipid complexes and SPLP were analyzed by Southern blot hybridization (Figure 3a, b and c, respectively) and quantified by phosphorimaging analysis (Figure 3d and e). For naked plasmid, less than 0.01% of the injected dose remained intact in the circulation at 15 min, and no intact tumor-associated plasmid could be observed at any time. For plasmid administered in complexes, only a small fraction (<2%) was still intact in the circulation at 15 min and less than 0.2% was found to be intact in tumor tissue at 1 h. In

Figure 1 Cryo-electron micrographs of (a) purified SPLP and (b) LUV prepared by extrusion. SPLP were prepared from DOPE:DODAC:PEG-CerC₂₀ (83:7:10; mol:mol:mol) and pCMVluc and purified employing DEAE column chromatography and density gradient centrifugation. LUV were prepared from DOPE:DODAC:PEG-CerC₂₀ (83:7:10; mol:mol:mol) by hydration and extrusion through filters with 100 nm diameter pore size. The arrows in panel (a) indicate the presence of residual 'empty' vesicles formed during the detergent dialysis process that were not removed by the density centrifugation purification step. The bar in panel (b) indicates 100 nm. For details of sample preparation and cryo-electron microscopy see Materials and methods.

Figure 2 Pharmacokinetics, tissue distribution and tumor accumulation of SPLP and plasmid DNA–cationic liposome complexes following intravenous administration in tumor-bearing mice as reported by the ^3H -CHE lipid marker. The levels of complexes (○) and SPLP (●) in the circulation, the lung, the liver and in Lewis lung tumor tissue are shown in panels (a), (b), (c) and (d), respectively. The accumulations in liver, lung and tumor were corrected for plasma contributions²⁹ and are expressed as a percentage of the total injected dose.

Table 1 Biodistribution of SPLP and plasmid DNA–cationic liposome complexes in mice 4 and 24 h following i.v. injection

Tissue	% Injected dose (s.e.m.)			
	SPLP		Complexes	
	4 h	24 h	4 h	24 h
Plasma	55.0 (1.7)	6.4 (1.0)	1.7 (0.2)	1.4 (0.3)
Liver	7.0 (0.6)	23.0 (4.3)	35.2 (2.3)	35.1 (3.5)
Lung	0.0 (0.1)	0.2 (0.1)	1.8 (0.8)	0.5 (0.0)
Spleen	0.4 (0.1)	1.6 (0.1)	0.2 (0.2)	0.1 (0.3)
Tumor	0.2 (0.0)	2.8 (0.5)	0.2 (0.1)	0.3 (0.2)

Both SPLP and complexes contained pCMVLuc as well as trace levels of the ^{14}C -labeled CHE lipid marker and were administered at a dose level of 100 μg plasmid per mouse. The biodistribution was measured employing the CHE lipid marker. s.e.m., standard error of the mean.

contrast, following i.v. injection of SPLP, approximately 85% of the injected plasmid DNA remained in intact form in the circulation at 15 min, and progressively higher levels of intact plasmid accumulated at the tumor site over the time-course of the experiment. The levels achieved at 24 h correspond to approximately 1.5% of the total injected plasmid DNA dose. The circulation half-life of intact plasmid DNA following injection of SPLP was calculated to be 7.2 ± 1.6 h, in good agreement with the circulation half-life of ^3H -CHE-labeled SPLP, confirming the highly stable nature of SPLP in the circulation.

Serum enzyme levels of alanine aminotransferase

(ALT) or aspartate aminotransferase (AST) were assayed for evidence of toxicity following i.v. administration of SPLP and plasmid DNA–cationic liposome complexes. Elevated ALT and AST levels are usually associated with liver damage, although elevated AST levels can also indicate systemic tissue damage. Mice receiving SPLP at dose levels as high as 175 μg plasmid DNA per mouse did not have significantly elevated serum levels of ALT and AST (Figure 4a). However, mice receiving doses of plasmid DNA–cationic liposome complexes corresponding to plasmid doses above 20 μg per mouse exhibited progressively higher serum levels of ALT and AST, reaching levels 100-fold above normal levels at plasmid doses of 75 μg (Figure 4b).

Intravenously administered SPLP promote gene expression in a distal tumor

It is of obvious interest to determine whether SPLP-mediated delivery of intact plasmid to the tumor site results in transgene expression at that site. Luciferase gene expression in tumor tissue was therefore monitored following i.v. injection of SPLP, naked plasmid DNA and plasmid DNA–cationic liposome complexes at dose levels corresponding to 100 μg plasmid DNA per mouse. This dose level corresponded to the maximum tolerated dose of complexes as evidenced by animal morbidity and mortality. As shown in Figure 5, administration of SPLP results in reporter gene expression at the tumor site, with maximum levels corresponding to 32 pg luciferase per gram of tumor tissue at the 48 h time-point and significant gene expression extending to 96 h after injection. Injection of free plasmid DNA or plasmid DNA–cationic liposome complexes, on the other hand, resulted in no detectable gene expression at the tumor site. It is of interest to note that i.v. administration of complexes did result in transfection in the lung, liver and spleen, whereas administration of SPLP did not result in detectable levels of gene expression in these organs (data not shown). In an attempt to understand why SPLP did not give rise to significant gene expression in the liver, the levels of intact plasmid in the liver 24 h after injection of SPLP into C57BL/6 mice (100 μg plasmid per mouse) bearing a subcutaneous Lewis lung carcinoma were analyzed by Southern blot hybridization. No intact plasmid could be detected in the liver whereas intact plasmid was readily detected at the tumor site (results not shown). This suggests that the ability of SPLP to transfect cells at the tumor site but not in the liver may reflect relatively rapid breakdown of SPLP and associated plasmid following uptake into liver phagocytes (Kupffer cells), which play a dominant role in clearing liposomal systems from the circulation.¹⁵ Lower gene expression in the liver may also reflect the finding that nonviral vectors such as SPLP transfect dividing cells much more efficiently than non-dividing cells¹⁶ or that Kupffer cells are less readily transfected than tumor cells.

Discussion

This study demonstrates that SPLP consist of plasmid DNA encapsulated in a bilayer vesicle, and that systemic administration of SPLP results in significant accumulation and transfection at a distal tumor site. There are three important features of these results. The first concerns the structure of SPLP, which represents a major

Figure 3 Pharmacokinetics and tumor accumulation of plasmid DNA following intravenous administration of naked plasmid, plasmid DNA–cationic liposome complexes and SPLP as reported by a Southern blot analysis. The Southern blot hybridizations shown in panels (a), (b) and (c) result from plasmid DNA isolated from blood and tumor tissue of mice injected with naked plasmid DNA, plasmid DNA–cationic lipid complexes and SPLP, respectively. Each panel shows pCMVluc (2 ng) to indicate the position of intact plasmid DNA. The levels of intact plasmid resulting from i.v. injection of naked plasmid DNA (□), plasmid DNA–cationic liposome complexes (○) and SPLP (●) were quantified for plasma (panel d) and tumor tissue (panel e) by phosphor-imaging analysis and converted to mass quantities of plasmid DNA by comparison to a standard curve made from known amounts of plasmid DNA dose.²⁹ Tumor accumulations of plasmid were corrected for plasma contributions and expressed as a percentage of the total injected plasmid DNA dose.²⁹

advance for plasmid encapsulation in liposomal delivery systems. Second, it is of interest to compare the properties of the SPLP system for systemic gene delivery and distal tumor transfection with the properties of other viral or nonviral gene delivery systems. Finally, the possi-

bilities for further optimization of the SPLP system are of interest. We discuss these areas in turn.

The cryo-electron microscopy results presented here establish the structure of SPLP as a plasmid surrounded by a lipid bilayer envelope. This represents the first direct

Figure 4 Toxicity resulting from i.v. injection into mice of varying amounts of SPLP (panel a) and plasmid DNA–cationic lipid complexes (panel b) as assayed by determining serum levels of the hepatic enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (ASP). The serum levels of AST (□) and ALT (■) were measured 24 h after injection.

demonstration that plasmid can be entrapped in small (diameter approximately 70 nm), well defined vesicular systems containing a single plasmid per vesicle. Entrapment of a plasmid such as pCMVluc, which contains 5650 bp, in a supercoiled configuration in a 70 nm diameter vesicle represents a solution for a difficult packing problem. For example, electron micrographs of supercoiled 4.4 kbp plasmids reveals extended lengths of approximately 500 nm and average (two dimensional) diameters in the range of 350 nm, suggesting an average diameter for free supercoiled pCMVluc of approximately 400 nm.¹⁷ The detergent dialysis process clearly involves a partial condensation of entrapped plasmid to allow encapsulation in a 70 nm diameter vesicle. The mechanism of entrapment is not understood in detail, but appears to proceed via association of plasmid with lipid structures formed as intermediates in the detergent dialysis process.¹²

SPLP exhibit extended circulation lifetimes ($t_{1/2}$ approximately 7 h) following i.v. injection, can deliver significant amounts of intact plasmid to a distal tumor site and

Figure 5 Transgene expression at a distal tumor site following intravenous injection of naked plasmid DNA (□), plasmid DNA–cationic lipid complexes (○) and SPLP (●). Mice bearing subcutaneous Lewis lung tumors were injected i.v. with doses containing 100 µg of pCMVluc. Tumors were harvested at the indicated times and assayed for luciferase activity. The level of transgene expression reported is normalized for the weight of the tumor tissue.

enable transgene expression corresponding to 30 pg luciferase per gram tumor at that site with no evidence of toxicity (as indicated by serum enzyme levels). Delivery of approximately 3% of the injected dose of SPLP at a 200 mg tumor site corresponds to more than 1000 plasmid copies per tumor cell, assuming a cell density of 1×10^9 per milliliter. It is of interest to compare these properties with the behavior of other gene delivery systems. In the case of viral vectors, there have been three reports of transgene expression in liver metastases and in a distal tumor following systemic administration of a recombinant vaccinia virus^{18,19} and a selectively replicating adenovirus.²⁰ These viral vectors are replication incompetent in normal nondividing cells but can selectively replicate in tumor cells resulting in transgene expression in tumors and antitumoral efficacy. The major drawback of these viral vectors is the immune response, which occurs within 6 days. In the case of nonviral vectors such as plasmid DNA–cationic polymer ‘polyplexes’, there is only one report showing transfection of distal tumors following i.v. injection.²¹ This work utilized a PEG-containing polyplex that exhibits plasmid circulation half-lives of less than 0.5 h following intravenous injection and gave rise to transfection at a distal tumor site, achieving transfection levels corresponding to approximately 250 pg/g tumor, approximately eight-fold higher than the levels reported here.

With regard to plasmid DNA–cationic liposome complexes (‘lipoplexes’), a number of studies have characterized transfection properties following i.v. administration,^{3–7} however, only two studies by Xu and co-workers have demonstrated transfection at a distal tumor site.^{22,23} In the initial study,²² less than 5% of the cells at the tumor site were transfected as indicated by immunohistochemical staining, whereas in the second study using transferrin targeted complexes 20–30% of the cells were transfected. The levels of gene expression could not be related to the levels observed here. Issues related to circulation lifetimes, plasmid tumor accumulation and toxicity were not addressed. An additional study²⁴ has demonstrated the presence of complexes at a distal tumor site following i.v. injection but the levels of

gene transfer were not measured. In general, i.v. injection of complexes gives rise to high levels of transgene expression in the lungs, with lower levels of expression in the spleen, liver, heart and kidneys. Similar results were observed for the complexes employed in this investigation. The lung expression appears to arise from deposition in lung microvasculature and reflects the rapid clearance of plasmid DNA–cationic lipid complexes from the circulation due to their large size (>200 nm diameter) and high cationic lipid content.⁸ This is consistent with the observation that murine B16 tumors seeded in the pulmonary vascular compartment can be transfected by i.v. administered complexes.³ Finally, as clearly shown in this study, administration of complexes is often associated with significant toxicity.

The final point of discussion concerns the utility of SPLP as a systemic gene therapy vector and the potential for further optimization. As indicated above, despite the delivery of large amounts of intact plasmid to the tumor site, the levels of gene expression observed for the SPLP system are modest, albeit comparable with or superior than can be achieved with other vectors. It is likely that the low levels of transfection reflect low levels of uptake of SPLP into cells at the tumor site due to inhibition of cell association and uptake by the PEG coating.²⁵ *In vitro* studies have shown that SPLP containing PEG-CerC₂₀ are accumulated into cells to a very limited extent, however, the SPLP that are taken up are highly transfection potent.²⁶ The challenge that faces the next stage of SPLP development is, therefore, to devise methods of enhancing intracellular delivery of SPLP following arrival at the tumor site. There are a number of avenues to explore. First, the dissociation rate of the PEG coating from the SPLP can be modulated by varying the acyl chain length of the ceramide anchor,¹² suggesting the possibility of developing PEG-Cer molecules that remain associated with the SPLP long enough to promote passive targeting to the tumor, but which dissociate quickly enough to allow transfection after arriving at the tumor site. Alternatively, improvements may be expected from inclusion of cell-specific targeting ligands in SPLP to promote cell association and uptake. Finally, the nontoxic properties of SPLP allow the possibility of higher doses. A dose of 100 µg plasmid DNA per mouse corresponds to a dose of approximately 5 mg plasmid DNA per kilogram body weight. This is a relatively low dose level in comparison to small molecules used for cancer therapy, which typically are used at dose levels of 10 to 50 mg per kg body weight.

In summary, we have shown that SPLP consist of plasmid encapsulated in a lipid vesicle. Furthermore, we have demonstrated that, in contrast to naked plasmid or complexes, SPLP exhibit extended circulation lifetimes following intravenous injection, resulting in plasmid accumulation and transgene expression at a distal tumor site in a murine model. The levels of transgene expression achieved are modest, but are comparable or superior to distal tumor expression levels achieved employing other vectors. Further improvements can be expected due to the low toxicity and flexible nature of the SPLP system.

Materials and methods

Lipids and plasmid

1,2-Dioleoyl-3-phosphatidylethanolamine (DOPE) was obtained from Northern Lipids (Vancouver, BC, Canada).

The cationic lipid N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC) and 1-O-[2'-(ω-methoxypolyethylene-glycol)succinoyl]-2-N-arachidoylsphingosine (PEG-CerC₂₀) were prepared at Inex Pharmaceuticals (Burnaby, BC, Canada) using previously described methods.²⁷ ³H-labeled cholesteryl hexadecyl ether (CHE) was purchased from Dupont NEN Products (Boston, MA, USA). The pCMVluc plasmid encodes the *Photinus pyralis* luciferase gene under the control of the human CMV immediate-early promoter. Plasmid DNA was propagated in *E. coli* (DH5α) and purified using the alkaline lysis method followed by two rounds of CsCl/ethidium bromide density equilibrium centrifugation.

SPLP and plasmid DNA–cationic liposome complex preparation

Plasmid DNA was encapsulated in SPLP composed of DOPE/DODAC/PEG-CerC₂₀ (83:7:10; mol:mol:mol) by the detergent dialysis method.¹² Lipids were dissolved in ethanol and dried to a lipid film in a round-bottom flask. The lipid mixture was resuspended in HBS (5 mM Hepes, 150 mM NaCl, pH 7.5) containing 200 mM OGP and 0.4 mg/ml pCMVluc. The final lipid concentration was 10 mg/ml. When required, ³H-CHE was added to a specific activity of 1.0 µCi/mg total lipid. The mixture of lipid, plasmid and OGP was dialyzed against 4 l of HBS for 2 days with three changes. Untrapped plasmid was removed by DEAE-Sepharose CL-6B chromatography, and plasmid DNA-containing SPLP were purified by sucrose gradient centrifugation (2.5%/5%/10%) in a Beckman SW 28 rotor (16 h at 107 000 g) (Beckman, Fullerton, CA, USA). DNA-containing particles banding at the 5%/10% sucrose interface were collected and concentrated by ultrafiltration before the DNA concentration was adjusted to 500 µg/ml. The final lipid composition was determined by HPLC analysis. DNA was quantified by picogreen (Molecular Probes, Eugene, OR, USA) fluorescence of TX-100-solubilized SPLP preparations. Plasmid DNA–cationic liposome complexes were prepared by adding pCMVluc to large unilamellar vesicles (LUV) composed of DOPE:DODAC (1:1; mol:mol) to a final charge ratio (+/–) of 3.0 in 5% glucose. The LUV were prepared by extrusion through 100 nm pore size filters according to standard procedures.²⁸

Cryo-electron microscopy

A drop of buffer containing SPLP was applied to a standard electron microscopy grid with a perforated carbon film. Excess liquid was removed by blotting leaving a thin layer of water covering the holes of the carbon film. The grid was rapidly frozen in liquid ethane, resulting in vesicles embedded in a thin film of amorphous ice. Images of the vesicles in ice were obtained under cryogenic conditions at a magnification of 66 000 and a defocus of –1.5 micron using a Gatan cryo-holder in a Philips CM200 FEG electron microscope (Eindhoven, The Netherlands).

Quasi-elastic light scattering

The mean diameter of SPLP was measured by quasi-elastic light scattering (QELS) using a Nicomp Model 370 Sub-Micron particle sizer (Santa Barbara, CA, USA) operated in the particle mode.

Clearance, biodistribution and tumor accumulation of SPLP

Lewis lung carcinoma cells (300000; ATCC CRL-1642) were implanted subcutaneously in the hind flank of 6-week-old female C57BL/6 mice (Harlan, Indianapolis, IN, USA) and the tumor allowed to grow to approximately 200 mg (12–14 days). Injected materials were then administered intravenously (lateral tail vein injection). All injected doses are reported in micrograms of plasmid DNA per mouse. Blood from animals was collected at the appropriate time-points into blood collection tubes by cardiac puncture. Tumors and organs were quickly removed and frozen at -70°C . Aliquots of plasma separated from blood were analyzed for ^3H -CHE by liquid scintillation counting. Plasmid DNA was purified by treating 50 μl plasma with $1\times$ proteinase K buffer (1.0 mg/ml proteinase K, 100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS, pH 8.0). After incubation at 37°C for 3 h, the samples were purified by phenol/chloroform extraction followed by ethanol precipitation. Tumors were homogenized in PBS containing 100 mM EDTA pH 8.0 by using the Fast-Prep 120 homogenizer system (Bio 101, Vista, CA, USA). DNA was purified from an aliquot of the tumor homogenates using the DNazol reagent according to the manufacturer's guidelines (Life Technologies, Bethesda, MD, USA). The DNA preparations from tumor homogenates were digested with *EcoRI*. DNA samples were subject to electrophoresis through 1.0% agarose gels, transferred to nylon membranes and subjected to Southern blot hybridization using a random primed ^{32}P -labeled restriction fragment from the luciferase gene. Hybridization intensity was quantified using a STORM840 phosphor-imager (Molecular Dynamics, Sunnyvale, CA, USA) and converted to mass of DNA using a standard curve constructed with known amounts of plasmid DNA.

Luciferase assays

Tumor tissue was homogenized in $1\times$ Cell Culture Lysis Reagent (CCLR) (Promega, Madison, WI, USA) using the Fast-Prep 120 homogenizer system (Bio 101). The homogenates were centrifuged at 10000 g for 2 min before 20 μl of the supernatant was assayed for luciferase activity using the Luciferase Assay System Kit (Promega) on an ML3000 microtiter plate luminometer (Dynex Technologies, Chantilly, VA, USA). Luciferase activities were converted to mass quantities of purified luciferase by comparison with a standard curve generated by assaying known amounts of purified *Photinus pyralis* luciferase enzyme (Boehringer-Mannheim, Laval, PQ, Canada) diluted into untreated tumor extract.

Hepatic release enzyme assays

Plasma from normal C57BL/6 mice injected with SPLP, plasmid DNA–cationic lipid complexes or HBS was recovered 24 h after injection by centrifugation and assayed immediately for ALT or ASP using commercially available kits (Sigma, St Louis, MO, USA).

Acknowledgements

We thank T Nolan, N Turcotte and J Johnson for excellent technical assistance, Dr P Joshi for assistance in preparing the manuscript and Dr S Ansell and Dr Z Wang for supplying the DODAC and PEG-CerC₂₀ respectively.

Plasmid DNA was prepared by C Giesbrecht and J Thompson.

References

- Huard J *et al*. The route of administration is a major determinant of the transduction efficiency of rat tissues by adenoviral recombinants. *Gene Therapy* 1995; **2**: 107–115.
- Worgall S, Wolff G, Falck PE, Crystal RG. Innate immune mechanisms dominate elimination of adenoviral vectors following *in vivo* administration. *Hum Gene Ther* 1997; **8**: 37–44.
- Zhu N, Liggitt D, Liu Y, Debs R. Systemic gene expression after intravenous DNA delivery into adult mice. *Science* 1993; **261**: 209–211.
- Thierry AR *et al*. Systemic gene therapy: biodistribution and long-term expression of a transgene in mice. *Proc Natl Acad Sci USA* 1995; **92**: 9742–9746.
- Li S, Huang L. *In vivo* gene transfer via intravenous administration of cationic lipid-protamine-DNA (LPD) complexes. *Gene Therapy* 1997; **4**: 891–900.
- Liu Y *et al*. Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery. *Nat Biotechnol* 1997; **15**: 167–173.
- Templeton NS *et al*. Improved DNA: liposome complexes for increased systemic delivery and gene expression. *Nat Biotechnol* 1997; **15**: 647–652.
- Huang L, Li S. Liposomal gene delivery: a complex package. *Nat Biotechnol* 1997; **15**: 620–621.
- Proffitt RT *et al*. Liposomal blockade of the reticuloendothelial system: improved tumor imaging with small unilamellar vesicles. *Science* 1983; **220**: 502–505.
- Gabizon A, Papahadjopoulos D. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc Natl Acad Sci USA* 1988; **85**: 6949–6953.
- Chonn A, Cullis PR. Recent advances in liposomal drug-delivery systems. *Curr Opin Biotechnol* 1995; **6**: 698–708.
- Wheeler JJ *et al*. Stabilized plasmid-lipid particles: construction and characterization. *Gene Therapy* 1999; **6**: 271–281.
- Mimms LT *et al*. Phospholipid vesicle formation and transmembrane protein incorporation using octyl glucoside. *Biochemistry* 1981; **20**: 833–840.
- Mayer LD *et al*. Comparison of free and liposome encapsulated doxorubicin tumor drug uptake and antitumor efficacy in the SC115 murine mammary tumor. *Cancer Lett* 1990; **53**: 183–190.
- Roerdink F *et al*. The involvement of parenchymal, Kupffer and endothelial cells in the hepatic uptake of intravenously injected liposomes. Effects of lanthanum and gadolinium salts. *Biochim Biophys Acta* 1981; **677**: 79–89.
- Mortimer I *et al*. Cationic lipid-mediated transfection of cells in culture requires mitotic activity. *Gene Therapy* 1999; **6**: 403–411.
- Lewis RJ, Huang JH, Pecora R. Rotational and translational motion of supercoiled plasmids in solution. *Macromolecules* 1985; **18**: 944–948.
- Gnant MFX *et al*. Sensitization of tumor necrosis factor α -resistant human melanoma by tumor-specific *in vivo* transfer of the gene encoding endothelial monocyte-activating polypeptide II using recombinant vaccinia virus. *Cancer Res* 1999; **59**: 4668–4674.
- Gnant MFX *et al*. Tumor-specific gene delivery using recombinant vaccinia virus in a rabbit model of liver metastases. *J Natl Cancer Inst* 1999; **91**: 1744–1750.
- Heise CC *et al*. Intravenous administration of ONYX-015, a selectively replicating adenovirus, induces antitumoral efficacy. *Cancer Res* 1999; **59**: 2623–2628.
- Ogris M *et al*. PEGylated DNA/transferring–PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Therapy* 1999; **6**: 595–605.
- Xu M *et al*. Gene therapy with p53 inhibits human breast tumors *in vivo* through a bystander mechanism without evidence of toxicity. *Hum Gene Ther* 1997; **8**: 177–185.

- 23 Xu L *et al*. Transferrin-liposome-mediated systemic p53 gene therapy in combination with radiation results in regression of human head and neck cancer xenografts. *Hum Gene Ther* 1999; **10**: 2941–2952.
- 24 Thurston G *et al*. Cationic liposomes target angiogenic endothelial cells in tumors and chronic inflammation in mice. *J Clin Invest* 1998; **101**: 1401–1413.
- 25 Lasic DD *et al*. Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. *Biochim Biophys Acta* 1991; **1070**: 187–192.
- 26 Mok KWC, Lam AMI, Cullis PR. Stabilized plasmid-lipid particles: factors influencing plasmid entrapment and transfection properties. *Biochim Biophys Acta* 1999; **1419**: 137–150.
- 27 Webb M *et al*. Comparison of hydrophobic anchors conjugated to poly(ethylene)glycol: effects on the pharmacokinetics on the liposomal vincristine. *Biochim Biophys Acta* 1998; **1372**: 272–282.
- 28 Hope MJ *et al*. Generation of multilamellar and unilamellar phospholipid vesicles. *Chem Phys Lipids* 1986; **40**: 89–107.
- 29 Bally MB, Mayer LD, Hope MJ, Nayar R. Pharmacodynamics of liposomal drug carriers: methodological considerations. *Liposome Technol* 1993; **18**: 27–41.