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Cisplatin encapsulated in phosphatidylethanolamine liposomes enhances the in vitro cytotoxicity and in vivo intratumor drug accumulation against melanomas

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Liposomes;
Phosphatidyle thanolamine;
Intratumor;
Melanoma

Summary

Background: Cisplatin is a potent anticancer drug for treating melanoma.
Objective: The aim of this study was to evaluate the possibility of using liposomes, for intratumoral distribution in a melanoma, composed of phosphatidylethanolamine (PE), for its cytotoxicity.
Method: The in vitro drug release, in vitro cytotoxicity against melanoma, and in vivo residence time in the tumor of liposome-encapsulated cisplatin were investigated. The liposomes were prepared and characterized in terms of their morphology, size, zeta potential, and drug loading.
Result: The size of the PE liposomes attained a level of ~100 nm. The concentration of cisplatin encapsulated in PE liposomes was 50—70% dependent on the presence or absence of polyethylene glycol (PEG) derivatives. On the other hand, no or negligible cisplatin molecules were encapsulated in egg phosphatidylcholine (EPC) liposomes. PE liposomes had higher cytotoxicity than classic liposomes or free cisplatin. Images of confocal laser scanning microscopy confirmed the great potency of PE liposomes to deliver cisplatin into cells. The incorporation of PEG derivatives completely inhibited the proliferation of melanoma cells. With in vivo intratumoral administration, the

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cisplatin concentration in the tumor tissue was maintained at a high level for 72 h after application of the PE liposomes. The PE liposomes delivered cisplatin into the tumor ~3.6 times more efficiently than the free drug.
Conclusion: These results demonstrate that PE liposomes represent a potentially useful strategy for targeting cisplatin delivery into melanomas.

1. Introduction

Cisplatin has long been widely used because of its broad spectrum of cytolytic activity against solid tumors including melanomas [1,2]. However, the full therapeutic exploitation of cisplatin is limited by its toxicity toward healthy tissues [3]. The selective delivery of cisplatin to tumor cells would significantly reduce drug toxicity, thus improving its therapeutic index [4]. Intratumoral administration to melanomas has been performed clinically to tackle these problems [5]. Intratumoral administration has been shown to achieve an ideally high concentration of drugs within the tumor. However, since a low-molecular-weight drug like cisplatin is rapidly passed into the blood circulation and the time period of retention in the tumor is very short, no significantly high and prolonged antitumor effects are expected [6]. To overcome this problem, drug-carrier systems such as liposomes are being investigated because of their favorable characteristics as a biodegradable drug reservoir to prolong retention times. This is especially important for melanomas since this tumor is highly resistant to chemotherapy and radiation therapy, and therapies for melanomas have generally been ineffective at best and have rarely resulted in sustained responses [7].

In most cases, conventional liposomes prepared for cisplatin offer low encapsulation efficacy and low drug loading [8,9]. Low cisplatin loadings have been obtained with other nanoparticle types [4,10]. The term "conventional liposomes" has a broad definition and refers to liposomes composed of a variety of different lipid compositions, but typically the most commonly used of these compositions are very high in phosphatidylcholine (PC) and cholesterol (CH) [11]. Hence finding other lipids for high-level encapsulation of cisplatin in liposomes is urgently needed. On the other hand, previous studies have suggested that phosphatidylethanolamine (PE) is easier to transfer across the membranes of mammalian cells than is PC [12,13]. This may contribute to the strong ability to carry cisplatin into cells. The aim of this study was to develop a novel liposome formulation composed of PE to circumvent the poor loading of cisplatin in conventional liposomes, thus strengthening the antitumor efficacy against melanomas. In vitro cell viability and in vivo drug deposition within melanomas were assessed to evaluate the efficacy of PE liposomes for intratumoral cisplatin administration. The physicochemical characteristics and drug release of PE liposomes were also determined.

2. Materials and methods

2.1. Materials

Cisplatin (*cis*-diammineplatinum (II) dichloride, $Pt(NH_3)_2Cl_2$), phosphatidylethanolamine (PE) from sheep brain, deoxycholic acid (DA), cholesterol (CH), and sulforhodamine B were purchased from Sigma Chemical (St. Louis, MO, USA). Egg phosphatidylcholine (EPC, 99%) as well as polyethylene glycol (PEG) derivatives of PE (PEP; with a mean molecular weight of PEG: 2000) and cholesterol (CP; with a mean molecular weight of PEG: 2000) were obtained from Nippon Oil (Tokyo, Japan). The melanoma cell line (B16-F0) was supplied by American Type Culture Collection (Rockville, MD, USA). All other chemicals and solvents were of analytical grade.

2.2. Preparation of liposomes

PE or EPC (160 mg) with DA (40 mg) and the other additives were dissolved in a 5-ml volume of a chloroform:methanol (2:1) solution. The reason why DA was incorporated in the systems was that DA can act as a stabilizer of PE bilayers [14]. The organic solvent was evaporated in a rotary evaporator at 50 °C, and solvent traces were removed by maintaining the lipid film under a vacuum for 6 h. The films were hydrated with double-distilled water containing 10 mg of cisplatin using a probe-type sonicator (VCX 600, Sonics and Materials, USA) at 25 W for 30 min. The total volume of the final formulations was 10 ml. The liposomal systems used in this study are listed in Table 1.

2.3. Transmission electron microscopy (TEM)

The liposomal vesicles were examined by TEM to characterize their microstructure. A drop of

Table 1 The composition and characterization of cisplatin liposomes by vesicle size, zeta potential, and drug encapsulation

Code	Composition[a]	Encapsulation (%)	Size (nm)	Zeta potential (mV)
F1	PE + CH (1.6:0.4)	70.0 ± 6.9	95.6 ± 3.8	−54.9 ± 5.3
F2	EPC + CH (1.6:0.4)	4.6 ± 1.2	148.7 ± 1.5	−7.1 ± 0.4
F3	PE + CP (1.6:0.4)	67.4 ± 8.3	82.7 ± 0.2	−63.8 ± 3.2
F4	PE + PEP + CP (1.2:0.4:0.4)	53.6 ± 6.4	97.7 ± 4.6	−41.0 ± 5.2

PE, phosphatidylethanolamine; CH, cholesterol; EPC, egg phosphatidylcholine; CP, cholesterol—PEG 2000; PEP, distearoyl phosphatidylethanolamine-PEG 2000. Each value represents the mean ± S.D. (n = 3).

[a] The ratio of liposome composition is weight ratio (%). All formulations contained deoxycholic acid (DA) with a concentration of 0.4% (w/w).

liposomes was applied to a carbon film-covered copper grid to form a thin film specimen, which was stained with 1% phosphotungstic acid. The sample was then examined and photographed with a Jeol JEM-1230 transmission electron microscope (Tokyo, Japan).

2.4. Determination of vesicle size and zeta potential

The mean vesicle size and zeta potential of the liposomes were measured by a laser scattering method (Nano ZS® 90, Malvern, UK). Liposomal suspensions were diluted 100-fold with double-distilled water before the measurement. The determination was repeated three times per sample for three samples.

2.5. Cisplatin encapsulation percentage in liposomes

The un-entrapped drug was removed by size exclusion chromatography on a Sephadex G-15 column using double-distilled water as the eluent. The encapsulation efficiency was determined by dissolving 500 μl of the liposomes obtained from gel chromatography in 500 μl of Triton X-100 (1%) and diluting this with double-distilled water (200×), followed by measurement of the absorbance by graphite furnace (flameless) atomic absorption spectrophotometry (AAS; Z-5000, Hitachi, Japan).

2.6. In vitro cisplatin release from liposomes

Drug release from liposomes was measured using a Franz diffusion cell. A cellulose membrane (Spectrapor® 3, with a molecular weight cutoff of 3500, Spectrum Laboratories, USA) was mounted between the donor and receptor compartments. The donor medium consisted of 1 ml of a liposomal formulation (with 1 mg cisplatin). The receptor medium consisted of 10 ml of pH 7.4 citrate—phosphate buffer. The available diffusion area between cells was 1.539 cm^2. The stirring rate and temperature were maintained at 600 rpm and 37 °C, respectively. At appropriate intervals, 300-μl aliquots of the receptor medium were withdrawn and immediately replaced with an equal volume of fresh buffer. The amount of drugs was determined by AAS.

2.7. Cytotoxicity assay

Melanoma cells were seeded at an initial concentration of 3×10^4 cells/well, and incubated in medium (10% fetal bovine serum, 89% DMEM, and 1% penicillin—streptomycin). Thirty microliters of liposomes with or without cisplatin diluted with medium (100 μM) was added at 7 h post-inoculation, and plates were incubated in a 5% CO_2 atmosphere at 37 °C for 12 and 24 h. After washing with PBS, cells were incubated with 5 mg/ml of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) in RPMI 1640 for 2 h at 37 °C. Formazan crystals resulting from MTT reduction were dissolved by adding 200 μl DMSO and gently agitating this for 30 min. The absorbance of the supernatant was then measured spectrophotometrically in an ELISA reader at 550 nm. Cell viability was calculated as the percentage of the control.

2.8. In vitro uptake of liposomes

Tumor cells (1×10^4 cells/well) were cultured for 7 h in a 24-well plate, then sulforhodamine B-labeled liposomes or the control solution (30 μl) were added for 1 h at 37 °C. Subsequently, melanoma cells were examined with a confocal laser scanning microscope (CLSM, Radiance 2100, Bio-Rad, CA, USA). Optical excitation was carried out with a 543-nm He—Ne laser beam, and the fluorescence emission was detected at 590 nm.

2.9. In vivo intratumoral administration

The solid tumors were obtained by a subcutaneous injection of 10^6 melanoma cells into the back of

female nude mice (ICR-Foxn/nu strain, 6 weeks old). The nude mice were purchased from the National Laboratory Animal Center, Taipei, Taiwan and were housed under pathogen-free conditions according to Chang Gung University animal care guidelines. All animal experiments were reviewed and approved by the Institutional Animal Care Committee at Chang Gung University. On day 7 as the size of the melanomas had grown to approximately 250 mm^3, 50 μl of liposomes (with 50 μg cisplatin) was intratumorally injected with a 29-gauge needle. At determined intervals, tumor sites were cut away and weighed for the following determination of cisplatin accumulation within tumors.

2.10. Extraction of cisplatin from solid tumors

After excising a tumor, it was weighed and minced with scissors, positioned in a glass homogenizer containing 1 ml of 4.31% sulfosalicylic acid, and ground for 5 min with an electric stirrer. The resulting solution was centrifuged for 10 min at 10,000 rpm and then filtered through a PVDF membrane (with a pore size of 0.45 μm, Millipore, USA). The drug amount in the supernatant was determined by AAS.

2.11. Statistical analysis

Statistical analysis of differences between different treatments was performed using unpaired Student's *t*-test. A 0.05 level of probability was taken as the minimal level of significance.

3. Results

3.1. Physicochemical characteristics of the liposomes

The physicochemical characteristics of the cisplatin-containing liposomes prepared with various phospholipids were investigated by assessing profiles of the drug encapsulation percentage, vesicle size, and zeta potential. The PE liposomes (F1) could efficiently entrap cisplatin into the vesicle based on the high encapsulation percentage of 70% shown in Table 1. EPC formulations (F2) showed no or negligible loading of cisplatin. In tumor tissues, drug release from liposomes may be accelerated by interstitial proteins and tumor-associated macrophages [15,16]. The PEG derivatives of PE and CH were incorporated into the liposomes (F3 and F4) to avoid this effect, since PEG on the vesicle surface serves as a barrier preventing interactions with proteins.

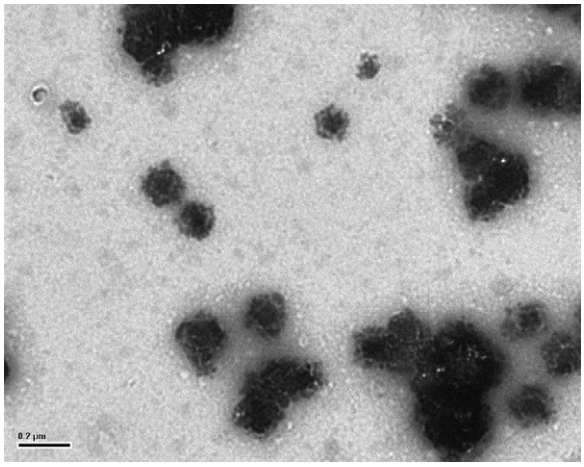

Fig. 1 Transmission electron microscopic micrograph of PE liposomes (F1). Original magnification 50,000×.

The incorporation of CP instead of CH did not alter the cisplatin entrapment in the liposomes (F1 versus F3, $p > 0.05$). However, cisplatin encapsulation was slightly but significantly reduced ($p < 0.05$) when PEP was added to the systems (F4).

The liposomes composed of PE (F1) were significantly smaller ($p < 0.05$) than those composed of EPC (F2) (Table 1). A picture of PE liposomes (F1) under TEM is shown in Fig. 1. The PE liposomal vesicles were spherical. The polydispersity index (size distribution) could be well controlled to a narrow range of ~0.3. This indicates a quite-homogeneous population of these vesicles. The incorporation of PEP and CP (F3 and F4) did not greatly change the vesicle size (Table 1).

The zeta potentials of the prepared liposomes are shown in Table 1. PE liposomes (F1) showed a highly negative surface charge (−54.9 mV). There was almost no surface charge on liposomes with EPC (F2). PEP shielded the electric surface potential of liposomes (F4).

3.2. In vitro cisplatin release from liposomes

The levels of in vitro cisplatin release from liposomes of different compositions are given in Fig. 2. The amount of cisplatin released from each liposomal system was plotted as a function of time. The aqueous solution of double-distilled water was used as the control. As shown in Fig. 2, the release of cisplatin from the aqueous solution showed an initial burst, then gradually leveled off after 4 h. EPC liposomes (F2) showed a similar release curve compared to the control. This may have been due to the negligible encapsulation of cisplatin in this system. Most of the cisplatin molecules were in the free form. The release of cisplatin from PE liposomes (F1)

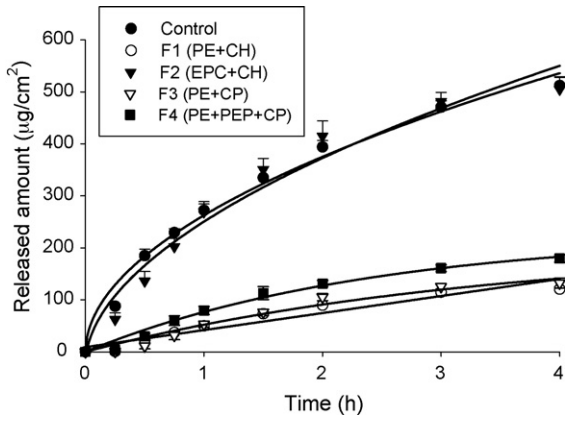

Fig. 2 In vitro release of cisplatin (measured as Pt by atomic absorbance) across a cellulose membrane from an aqueous solution (control) and liposomes with various compositions. Each value represents the mean and S.D. ($n = 4$).

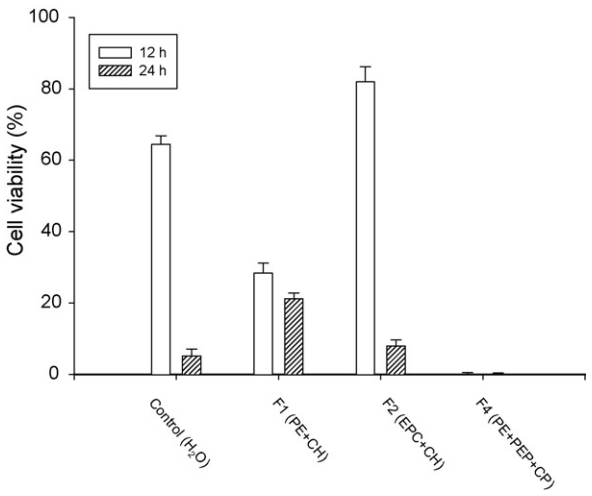

Fig. 3 Viability of melanoma cells (B16-F0) following treatment with cisplatin (100 μM) in an aqueous solution (control) or liposomes after incubation for 12 and 24 h. Each value represents the mean and S.D. ($n = 4$).

was significantly lower than those determined from liposomes with EPC. The addition of CP instead of CH did not significantly change cisplatin release (F3 versus F1). PEP slightly but significantly accelerated cisplatin release ($p < 0.05$) from PE liposomes (F4 versus F3).

3.3. Cytotoxicity assay

To evaluate the efficacy of liposomes for cisplatin delivery to melanomas, the in vitro cytotoxicity of PE liposomes was examined in comparison with EPC liposomes and free cisplatin. Since F3 showed similar drug release profiles to F1, three liposomal formulations (F1, F2, and F4) were selected to determine their cytotoxicity against melanomas. The effects of liposomes on the viability of melanomas studied after 12- and 24-h incubation periods are presented in Fig. 3. The cytotoxicity showed a period-dependent effect. Twelve-hour administration was not sufficient for free cisplatin (control) to induce apoptosis in melanoma cells. A strong inhibition of cell proliferation, however, was observed with free cisplatin 24 h after administration. Cisplatin loaded in PE liposomes (F1) showed higher in vitro cytotoxic activity ($p < 0.05$) than the free drug in the 12-h incubation experiment. However, a contrary result was observed in the 24-h incubation experiment. EPC liposomes exhibited almost no cytotoxicity against melanomas at 12 h. The antiproliferative activity of EPC liposomes was less than that of the control at 24 h. It is surprising that cisplatin loaded in PEP-containing liposomes (F4) could completely inhibit the proliferation of melanoma cells at both 12 and 24 h. Empty liposomes without cisplatin were also tested, and it was found that the vesicles themselves had no effect on cytotoxicity (data not shown). This indicates that the cytotoxicity toward the melanomas was mainly a consequence of the cisplatin molecules.

3.4. In vitro uptake of liposomes

Liposomes need to enter the cells and diffuse through the viscous cytosol to access the particular cytoplasmic targets where the sites of action are located [17]. To determine whether liposomes can be internalized by tumor cells, the uptake of sulforhodamine B-labeled liposomes (F1 and F2) and free sulforhodamine B in aqueous solution (control) was examined. After 1 h of incubation followed by washing, melanoma cells showed negligible fluorescence from the aqueous solution as shown in Fig. 4B. The left sides of Fig. 4 show the morphology of melanoma cells under a white light. CLSM showed that PE liposomes entered melanoma cells in a greater quantity (Fig. 4D). When cells were incubated with EPC liposomes, they showed no fluorescence (Fig. 4F). Enlarging the field of view in the image of the PE liposome-treated group revealed that almost all melanoma cells had successfully been invaded by sulforhodamine B (Fig. 4G and H).

3.5. In vivo intratumoral administration

Cisplatin by itself or incorporated within liposomes was intratumorally injected into melanomas on the backs of nude mice to examine drug deposition. Fig. 5 shows the retention of cisplatin in melanomas at different periods after the injection. Cisplatin accumulation within the melanomas was low in the free drug form (control). A similar profile was

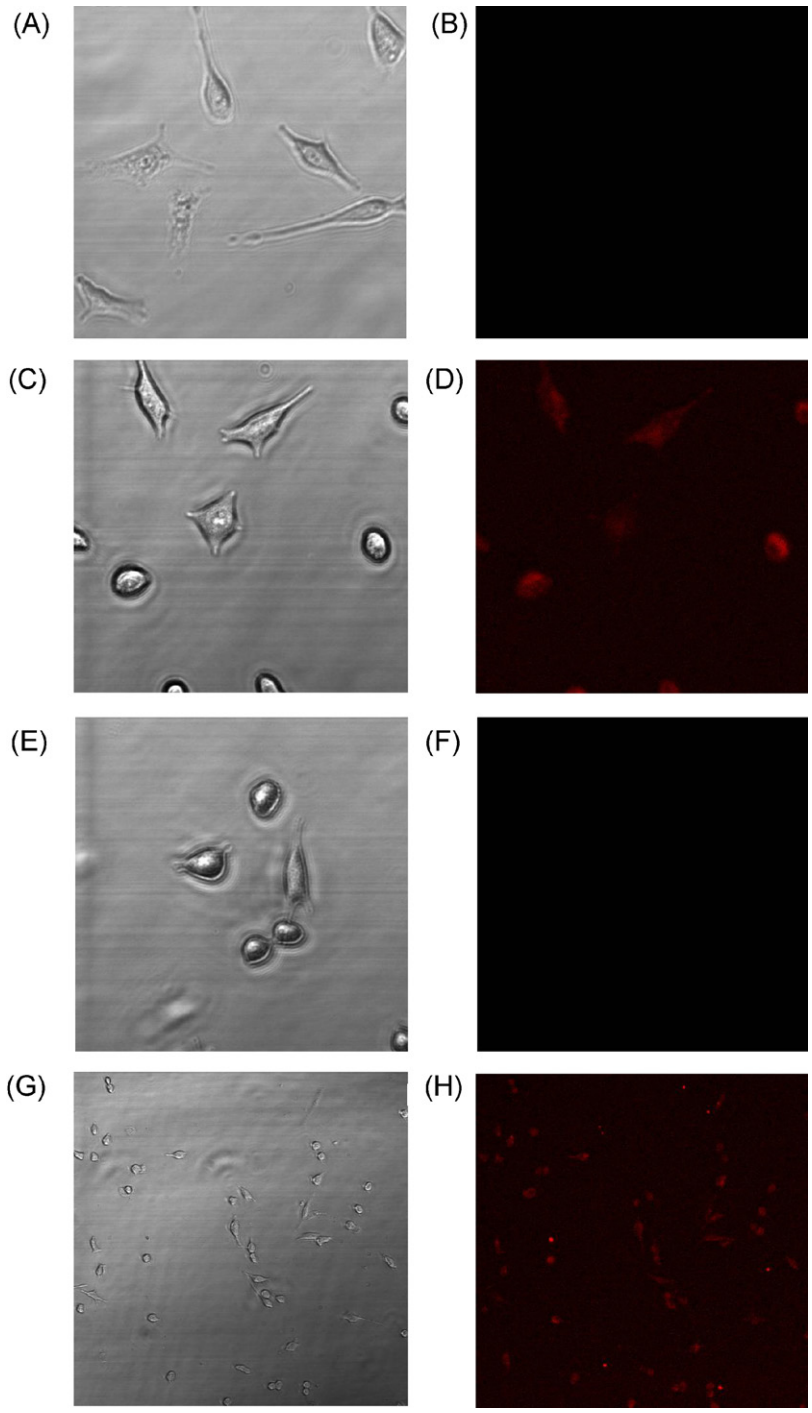

Fig. 4 Confocal laser scanning microscopic images of uptake of sulforhodamine B-labeled liposomes by melanoma cells. (A) Melanoma cells treated with an aqueous solution (control) observed under a white light; (B) melanoma cells treated with an aqueous solution (control) observed under a He—Ne laser beam; (C) melanoma cells treated with PE liposomes (F1) observed under a white light; (D) melanoma cells treated with PE liposomes (F1) observed under a He—Ne laser beam; (E) melanoma cells treated with EPC liposomes (F2) observed under a white light; (F) melanoma cells treated with EPC liposomes (F2) observed under a He—Ne laser beam; (G) melanoma cells treated with PE liposomes (F1) observed under a white light with an enlarged field of view; (H) melanoma cells treated with PE liposomes (F1) observed under a He—Ne laser beam with an enlarged field of view.

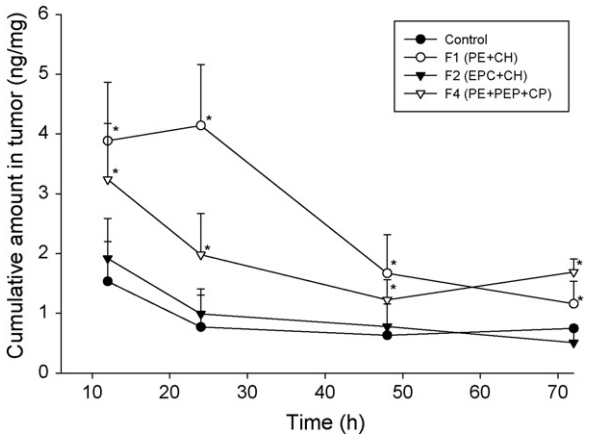

Fig. 5 In vivo tumor uptake of cisplatin (measured as Pt by atomic absorbance) for various durations from an aqueous solution (control) and various liposome systems (F1, F2, and F4) after an intratumor injection into melanomas on the backs of nude mice. Each value represents the mean and S.D. ($n = 4$).

detected for EPC liposome-encapsulated cisplatin (F2). The entrapment of cisplatin in liposomes with PE (F1 and F4) resulted in a significant increase in the cisplatin presence in tumors. Among all the applied groups, PE liposomes without PEG showed the most superior effect on the increase in cisplatin accumulation within the melanomas. When PE liposomes were used, a significantly higher level was detected in tumor tissue for 72 h after application, although the level tended to decrease with time. This suggests that the drug was metabolized and/or escaped from the tumor over time. Cisplatin exposure in the tumor according to the area under the curve (AUC) of the cumulative amount—time profiles was 2.3- and 3.6-fold higher compared to the control following the administration of PE liposomes with and without PEG derivatives, respectively.

4. Discussion

It is apparent that cisplatin entrapment in PE liposomes (F1) was significantly higher (15-fold) than that in EPC liposomes (F2). The gel to liquid-crystalline phase transition (T_m) takes place at about 36 and $-2.5\ ^\circ C$ for PE and EPC, respectively [11,18]. Bilayer rigidification using high-T_m phospholipids reduces the drug leakage from liposomes [19]. The reverse-phase evaporation method of preparing liposomes used in this study is based on the passive entrapment of the drug during formation of the lipid bilayer vesicles, resulting in a low encapsulation efficacy [9]. Hence the high cisplatin loading in PE liposomes may also be attributed to interactions between cisplatin and the phospholipid bilayers.

Phosphatidylcholine (PC) is the most commonly used phospholipid for preparing liposomes. PE contains an ethanolamine head group instead of the choline head group on PC. There are lone paired electrons on the nitrogen atom in the ethanolamine head group, but they are absent from the choline head group. Cisplatin is a platinum-chelated complex with four ligands: two ammonias and two chlorides. According to previous studies [20,21], the two chloride ligands are gradually substituted with H_2O molecules in water. Both $Pt(NH_3)_2Cl(OH_2)^+$ and $Pt(NH_3)_2Cl(OH_2)_2^{2+}$ can be formed by the aqueous complex based on the cisplatin dose used in liposomes prepared in this study. It may be easy for the aqueous complex to form a chelate with the lone paired electrons in PE. This phenomenon could not be occur with PC because of the lack of the lone paired electrons and the steric barrier produced by the larger head group.

In order to elucidate this mechanism, a derivative of cisplatin, carboplatin, was encapsulated into PE liposomes (F1). Carboplatin is produced by substituting chlorides in cisplatin with the ligand of 1,1-cyclobutane dicarboxylate. Hence carboplatin cannot form the aqueous complex. The encapsulation percentage of carboplatin only showed a value of $2.49 \pm 0.80\%$. This suggests the importance of the interaction between cisplatin and the PE bilayers. The incorporation of PEP reduced the cisplatin entrapment in liposomes. This is possible since the presence of PEG may decrease the stability of some liposomal formulations [11,22].

PC is a neutral or zwitterionic phospholipid over a pH range from strongly acidic to strongly alkaline. The EPC used in this study contained 99% PC. The other components (1%) of EPC included phosphatidylserine, phosphatidic acid, phosphatidylglycerol, and phosphatidylinositol, all of which are negatively charged [23]. These anionic fractions and the presence of DA were responsible for the -7.1 mV surface charge of the EPC liposomes. On the other hand, the charge of PE is negative at a pH of near 7.0 [24]. This pH approximated the value of PE liposomes (pH 6.7). This may have contributed to the highly negative charge of PE liposomes (-54.9 mV). The profiles of the zeta potential may explain the smaller size of PE liposomes compared to EPC liposomes, since the negatively charged phospholipids tend to increase the interbilayer distance owing to electrostatic repulsive forces, which avoid aggregation among vesicles [25]. The addition of PEP to the bilayers led to initial decreases in the zeta potential. PEP may reside on the surface of the bilayers because of its hydrophilicity. This may result in a shielding of the negative surface charge provided by the phospholipids.

For development of liposomes encapsulating antitumor agents in an in vivo status, it is important to optimize the ability to release the drug from the vesicles. The drug may be stably retained in the vesicles for a determined duration, followed by its slow release into the external phase. As shown in Fig. 2, the composition of liposomes significantly affected the rate of cisplatin release. The slow release of cisplatin from PE liposomes was attributed to the high entrapment and chelating interaction of cisplatin within the liposomal bilayers. The addition of PEP slightly increased cisplatin release, which may have been due to the lower cisplatin encapsulation in vesicles compared to liposomes without PEP (Table 1).

The ultimate goal of cisplatin delivery can be fulfilled only if it is able to localize and integrate with DNA. The aqueous complex of cisplatin is poorly permeable through cell membranes [20], contributing to its low cytotoxicity against melanomas at 12 h in this study. PE liposomes enhanced the cytotoxicity over a short period (12 h). Cisplatin was slowly released from the vesicles of PE liposomes. Hence this high activity against melanomas was not due to the direct penetration of free cisplatin into the cytoplasm. Cellular uptake of cisplatin can possibly occur by an endocytotic pathway of vesicles or by fusion of liposomal surfaces with the cell membrane. Although there seem to be several mechanisms responsible for liposome internalization, several lines of evidence suggest that endocytosis is the major way [26].

Since EPC produced much less cytotoxicity and cellular uptake than PE, the difference between these two phospholipids should be discussed. One of the major differences between PE and PC is the high non-bilayer-forming activity of PE and the absence of this activity with PC [13]. PE is a phospholipid which exhibits a high tendency to form the inverted hexagonal phase particularly at an acidic pH. Protonation of the head group of PE, caused by a reduction of the pH in physiological conditions, neutralizes the negative charge, and the vesicles become destabilized as the PE component reverts to the hexagonal phase. Fusion of the biological membrane or endocytosis may involve this inverted structure. Previous studies suggested the excellent ability to facilitate drug or gene delivery into cells by PE [27,28]. Another mechanism of PE uptake by cells may be the transbilayer internalization pathway [12]. Once PE is located on the inner leaflet of the plasma membrane, it is able to move to other intracellular membranes, presumably by spontaneous or facilitated transfer of this phospholipid. This phenomenon was not be detected with PC [12].

Much attention has been drawn to the use of 1, 2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) liposomes, which most often contain positively charged lipids, for drug delivery and gene transfection [14,29]. This is because the cationic surface of liposomes is necessary for cell binding prior to internalization by endocytosis. This was not the case in the present study, since PE liposomes possessed a strong negative charge. The effect of the surface charge on PE liposomes thus could be ruled out in the case of cisplatin delivery. Cisplatin loaded in EPC liposomes even showed weaker cytotoxicity against melanomas compared to the aqueous solution. The low encapsulation of cisplatin in EPC liposomes contributed to the existence of both free cisplatin and blank vesicles in this system. The presence of blank vesicles may have retarded the permeation of cisplatin into cells.

Liposomes targeting tumor cells have shown considerably greater cytotoxicity. This is due to an increased bioavailability after transport of liposomes to cytoplasm, where degradative enzymes break down the liposomal membrane and release the drug [11]. That is why PE liposomes exhibited increased cisplatin cytotoxic activity after a 12-h incubation. The cytotoxicity of PE liposomes was maintained at the same level ($p > 0.05$) from 12 to 24 h. This indicates that the endocytosis of PE liposomes occurred for only a limited duration. The presence of PE liposomes even retarded the antiproliferative activity of cisplatin compared to the control at 24 h. This phenomenon was not observed in PEP-containing liposomes. Almost no cell viability was detected after treatment with PEP-containing liposomes at either 12 or 24 h.

The strong in vitro cytotoxicity of PEP-containing liposomes was surprising because the endocytosis of liposomes conjugated with PEG by tumor cells does not normally occur to a significant extent [30]. PEP and/or CP are likely to provide suitable targets for liposome binding and internalization by melanoma cells in PE liposomal systems. This effect could remain for a longer period of at least 24 h. The mechanisms of action with PEP-containing liposomes remain open and need to be further explored.

A previous study demonstrated that cisplatin encapsulated in cationic PC liposomes with PEG only showed a limited prolongation of the mean survival time of mice because of the limited penetration of these long-circulating liposomes into the interior of established solid tumors [31]. Hence the direct injection of liposomes into tumor tissues is necessary with cisplatin. The challenge in drug targeting is not only to target a drug to a specific site but also to retain it for a desired duration to elicit pharmacological actions [17]. Tumors have large interstitial

spaces composed predominantly of a collagen and elastic fiber network. The vascular structure inside tumors is leaky [26]. A relatively low accumulation of cisplatin in melanomas was achieved with the aqueous solution. This is because the free drug with a small molecular mass rapidly diffuses away from tumor tissues [32]. By delivering cisplatin to melanomas with PE liposomes, the encapsulated cisplatin could efficiently be localized at the tumor site. Larger liposomes may be more-readily trapped by the fiber network, and the leakiness of capillary walls may be insufficient to allow their efflux from the interstitial into the vascular space. A liposomal size of $\sim$100 nm is thought to be suitable for its being trapped in the tumor interstitium [15,33]. The size of our PE liposomes (F1 and F4) may have been within this preferred range. The vesicle surface of PE liposomes was highly negatively ionized. The negatively charged glycocalyx in the vascular walls may repel PE liposomes [34], thus retaining these liposomes within the tumor interstitium.

The addition of PEG derivatives in PE liposomes reduced the cisplatin accumulation in tumors. A possible reason was the lower cisplatin encapsulation and quicker drug release of PEP-containing liposomes. Liposomes with rigid bilayers show superior tumor accumulation [15]. The inclusion of CH can strengthen the bilayer rigidity of liposomes. The substitution of CP to CH in the liposomes with PEG derivatives may weaken this rigidity, leading to a lower tumor deposition of cisplatin. Another possible reason is that the hydrophilic coating of the liposomal surfaces with PEG was not suitable for the interstitial environment of melanomas, increasing its escape from tumor tissues. This result may create a conflict since the original aim of adding PEG derivatives to the liposomes was to inhibit the clearance by interstitial proteins and tumor-associated macrophages [15,16].

5. Conclusions

The purpose of this study was to assess the feasibility of using PE liposomes to promote the therapeutic benefits of cisplatin against melanomas. Encapsulation of cisplatin in PE liposomes was able to overcome many of the limitations experienced with other liposomal formulations. The entrapment efficiency of cisplatin was significantly increased, the vesicle size was reduced, and cisplatin release from PE liposomes could be controlled. It is obvious that PE liposomes encapsulating cisplatin have great potential cytotoxicity against melanomas. The antitumor effect of PE liposome-loaded cisplatin was significantly higher than that of the free drug. The incorporation of PEG derivatives in PE liposomes further increased the cytotoxicity. As toxicity to normal cells has been reported with commonly used cationic liposomes such as DOTAP, research into reducing the toxicity of these carriers is required. PE liposomes with negative surface charges may overcome this drawback. The incorporation of cisplatin into PE liposomes greatly increased drug uptake by melanomas with intratumoral administration. It is probable that the increased cisplatin encapsulation, enhanced cytotoxicity against melanomas, and targeted delivery of PE liposomes in the tumors accounted for the enhanced therapeutic effect of the liposomal cisplatin.

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