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In vivo distribution and antitumor activity of heparin-stabilized doxorubicin-loaded liposomes

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Abstract

The purpose of this study was to investigate the effect of heparin conjugation to the surface of doxorubicin (DOX)-loaded liposomes on the circulation time, biodistribution and antitumor activity after intravenous injection in murine B16F10 melanoma tumor-bearing mice. The heparin-conjugated liposomes (heparin-liposomes) were prepared by fixation of the negatively charged heparin to the positively charged liposomes. The existence of heparin on the liposomal surface was confirmed by measuring the changes in the particle size, zeta potential and heparin amount of the liposomes. The stability of the heparin-liposomes in serum was higher than that of the control liposomes, due to the heparin-liposomes being better protected from the adsorption of serum proteins. The DOX-loaded heparin-liposomes showed high drug levels for up to 64 h after the intravenous injection and the half-life of DOX was approximately 8.4- or 1.5-fold higher than that of the control liposomes or polyethyleneglycol-fixed liposomes (PEG-liposomes), respectively. The heparin-liposomes accumulated to a greater extent in the tumor than the control or PEG-liposomes as a result of their lower uptake by the reticuloendothelial system cells in the liver and spleen. In addition, the DOX-loaded heparin-liposomes retarded the growth of the tumor effectively compared with the control or PEG-liposomes. These results indicate the promising potential of heparin-liposomes as a new sterically stabilized liposomal delivery system for the enhancement of the therapeutic efficacy of chemotherapeutic agents.

Keywords: Heparin conjugation; Liposome; Circulation time; Biodistribution

1. Introduction

Liposomes have been extensively investigated as carriers for a variety of drugs including the anticancer drug, doxorubicin (DOX) (Eliaz et al., 2004; Drummond et al., 1999). Liposomal drugs are usually injected intravenously for systemic applications. However, liposomes have been found to be plagued by rapid opsonization and by their being taken up by the reticuloendothelial system (RES) cells located mainly in the liver and spleen. In general, this rapid uptake of the liposomes leads to their having a short circulation time. This problem has been resolved by incorporating lipid-grafted polyethyleneglycol (PEG) into the liposome membrane. The incorporation of lipid-grafted PEG reportedly reduced the opsonization of the liposomes and consequently increased their circulation time (Allen et al., 1991; Mercadal et al., 1999; Lu et al., 2004; Papahadjopoulos et al., 1991). In spite of these advances, however, up to now the use of clinically approved liposomal formulations has only resulted in a modest increase in the therapeutic efficacy of anticancer drugs.

To overcome these delivery concerns associated with the short circulation time of liposomes in the field of chemotherapy, a number of liposomes with various functionalities have been designed (Yatvin et al., 1978; Needham and Dewhirst, 2001; Xiong et al., 2005a). One of the approaches employed to prolong the circulation time of the liposomes is polymer conjugation on their surface (Sadzuka et al., 2002; Metselaar et al., 2003; Auguste et al., 2003; Takeuchi et al., 1999). Polymer fixation is a simple and highly efficient method for the conjugation of polymer to liposomes, which is accomplished simply by mixing

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the liposomal suspension with the polymer solution, and occurs without covalent bonding of the polymer to the lipid molecule (Sahli et al., 1998).

Heparin is one of the most potent anticoagulants and is widely used for the treatment and prevention of deep vein thrombosis or pulmonary embolism (Damus et al., 1973; Lee et al., 2001). Heparin is a negatively charged polysaccharide and its hydrophilic properties make it difficult for heparin molecules to penetrate through the epithelial cells, due to its low permeability and the repulsion forces against the polar head group of the epithelial membrane (Norris et al., 1998). However, the conjugation of heparin to the liposomal surface, in order to prolong the circulation time and hence improve the antitumor potency of liposomes, has not been sufficiently studied.

The objective of this study was to evaluate whether the enhancement of the circulation longevity or antitumor activity of DOX-loaded liposomes could be achieved by conjugating heparin to the liposomal surface. The in vitro stability of heparin-conjugated liposomes (heparin-liposomes) was investigated and compared with those of PEG-fixed liposomes (PEG-liposomes) and control liposomes. The pharmacokinetics, biodistribution and therapeutic efficacy of these liposomal DOX formulations were investigated using murine B16F10 tumor-bearing mice.

2. Materials and methods

2.1. Materials

Dimethyldioctadecylammonium bromide (DDAB), L-α-phosphatidylcholine(soy-hydrogenated) (HSPC), cholesterol (CHOL) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000] (DSPE-mPEG-2000) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Doxorubicin (DOX) as an anticancer drug was obtained from Boryung Pharm. Co. (South Korea). Heparin (low molecular weight, 3000 Da) was purchased from Sigma Co. (Alabaster, AL, USA). Aqueous fetal bovine serum (FBS) and Dulbecco's modified Eagle medium (DMEM) were purchased from Bio-Tech. Inc. (Parker Ford, USA) and Invitrogen Inc. (CA, USA), respectively. Toluidine blue was purchased from Sigma Co. (Alabaster, AL, USA). All other materials were of analytical grade and used without further purification.

2.2. Preparation of liposomes

The DOX-loaded liposomes were prepared according to the remote loading method using an ammonium sulfate gradient (Haran et al., 1993; Lasic et al., 1995). The prepared liposomes and their lipid compositions were as follows: (1) control liposomes; HSPC:CHOL = 7:3 (molar ratio, total 10 mmol); (2) PEG-liposomes as DOXIL® formulation; HSPC:CHOL:DSPE-mPEG-2000 = 9.58:3.19:3.19 mg/ml; (3) positively charged liposomes to conjugate heparin; HSPC:CHOL:DDAB = 7:3:2 (molar ratio, total 10 mmol). Briefly, the lipids with the above compositions were dissolved in chloroform, dried into a thin film on a rotary evaporator (Buchi Rotavapor R-200, Switzerland) and then suspended in 250 mM ammonium sulfate solution. The liposomal solution was extruded through a polycarbonate filter (pore size; 100 nm, Whatman, USA) using an extruder (Northern Lipids Inc., USA). The free ammonium sulfate was removed by cellulose dialysis tubing (MWCO 3500, Viskase Co., IL, USA) for 24 h at 4 °C. The liposomal solution and 1 mg/ml DOX solution (1.7 mM in 10% sucrose solution) were mixed and then incubated for 2 h at 60 °C. The mixture was dialyzed to remove the free DOX. The DOX-loaded liposomes were stored at 4 °C until use. The concentration of DOX in the liposomes was measured by UV–vis spectrophotometry at 490 nm (UV-mini, Shimadzu, Japan) and the loading efficiency was calculated according to the following equation:

$$\text{Loading Efficiency}\,(\%) = F_i/F_t \times 100 \quad (1)$$

where F_i is the concentration of DOX loaded in the liposomes after their dissolution in 10% Triton X-100 and F_t is the initially added concentration of DOX.

To conjugate the heparin on the surface of the positively charged liposomes, the liposomes were incubated in heparin solution (1–9 mg/ml) at 25 °C for 2 h. The heparin-liposomes were isolated by gel filtration using a sephacryl-400 column at 4 °C in PBS solution (pH 7.4). The particle size and zeta potential of the liposomes were measured by light scattering with a particle size analyzer (ELS-8000, Otuska, Japan).

The amount of heparin on the liposomal surface was measured using the Toluidine blue assay (Wirsen et al., 1996). Briefly, in order to label Toluidine blue on the sulfate group of the heparin, 0.1 ml of 0.25 mg/ml Toluidine blue aqueous solution was added to 1 ml of the heparin-liposomes suspension and then reacted for 1 h at room temperature. The absorbance was measured at 630 nm using UV–vis spectrophotometry. The total amount of heparin on the surface of the liposomes was determined using a calibration curve of Toluidine blue-labeled heparin solution.

2.3. Stability assay of liposomes in serum

The protein adsorption on the surface of the liposomes is an important index for the stability of liposomes in serum. The adsorption of the serum proteins on the surface of the liposomes was evaluated by measuring the change in the size of the liposomes (Sahli et al., 1998; Lin et al., 1997). One milliliter of liposomal solution was added to 1 ml of 50% (v/v) serum and the samples were incubated at 37 °C with mild stirring. The size of the liposomes was measured by light scattering with a particle size analyzer (ELS-8000, Otuska, Japan).

2.4. Cell line and animals

B16F10, a murine melanoma cell line, was cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 10 μl/ml penicillin–streptomycin. The cultures were sustained at 37 °C in a humidified incubator containing 5% CO_2. The cells were maintained within their exponential growth phase.

Female C57BL/6 mice (5–6 weeks old, 18–22 g) were purchased from Harlan Int. (IN, USA). All of the procedures involved in the animal experiments were performed according to approved protocols and in accordance with the recommendations of the NIH guideline for the proper use and care of laboratory animals.

2.5. Pharmacokinetics study

In order to monitor the plasma levels of DOX in mice, free or liposomal DOX was injected via a tail vein at a dose of 6 mg DOX/kg body weight. At a predetermined time after the intravenous (i.v.) injection, mice from each group were sacrificed. Blood was collected immediately by cardiac puncture of the mice. Two hundred microliters of blood were collected and mixed with an equal volume of normal saline. After the centrifugation of the blood, the serum was divided into two equal aliquots. To the first aliquot were added 250 μl of 66 mM EDTA solution and isotonic 50 mM PBS buffer (pH 7.4) to make a final volume of 1 ml. The concentration of DOX was measured using UV–vis spectrophotometry at 490 nm. To the second aliquot were added 250 μl of EDTA solution, 150 μl of Triton X-100 solution and isotonic PBS buffer to make a final volume of 1 ml. The amount of DOX entrapped in the liposomes was calculated from the difference in the absorbance of the two aliquots. The pharmacokinetic parameters were calculated from the average DOX concentrations in the bloodstream using a pharmacokinetic software BA calculator (Seoul National University, South Korea). The significance of the in vivo data was evaluated using the Student's *t*-test and $P < 0.05$ was considered statistically significant.

2.6. Tissue distribution study

In the biodistribution study, 5×10^5 cells of murine B16F10 melanoma in 20 μl were carefully inoculated into the right limb armpit of the mice subcutaneously. The mice (three per group) were monitored for 64 h after an i.v. injection via the tail vein at a dose of 6 mg DOX/kg body weight. At a predetermined time after the i.v. injection, the mice were sacrificed and their liver, spleen, heart and tumor were collected immediately. The organs and tumor were carefully washed with distilled water, weighed and homogenized with PBS buffer solution (pH 7.4). The DOX concentration in the homogenized tissue was measured using UV–vis spectrophotometry at 490 nm, as described in Section 2.5.

2.7. Antitumor activity

A 5×10^5 cells of murine B16F10 melanoma in 20 μl were carefully inoculated into the right limb armpits of the mice subcutaneously. Six days after the tumor inoculation, free DOX solution or liposomal DOX suspension was injected intravenously via a tail vein at a dose of 6 mg DOX/kg body weight. The tumor volume was monitored for 16 days after a single i.v. injection of various liposomal formulations. In order to determine the tumor volume, each individual tumor size was measured with a caliper and the tumor volume was calculated using the following equation:

$$\text{Tumor volume (mm}^3\text{)} = \text{width} \times \text{length}^2/2. \quad (2)$$

3. Results

3.1. Characteristics of heparin-liposomes

The structural consequences of various liposomes were evaluated by measuring their particle size and zeta potential and the results are summarized in Table 1. The mean particle size of the control liposomes, PEG-liposomes and heparin-liposomes was approximately 90–100 nm and the loading efficiency of DOX was 89–91%, indicating that all of the liposomes had similar physicochemical characteristics. In addition, the zeta potential of the positively charged liposomes was 14.02 ± 2.6 mV and this value changed to -70.12 ± 0.7 mV as a result of the fixation of the negatively charged heparin. These results confirmed that heparin could be completely fixed on the surface of the liposomes.

The amount of heparin on the liposomal surface was determined by measuring the absorbance of Toluidine blue dye-labeled heparin. As shown in Fig. 1, the amount of heparin on the liposomal surface increased as the heparin concentration increased from 1 to 5 mg/ml. The amount of heparin on the surface of the liposomes reached 2.45 ± 0.2 mg/ml at a heparin concentration of 5 mg/ml and then leveled off when the heparin concentration was further increased. These results indicate that the surface of the positively charged liposomes could be fully fixed with heparin by ionic interaction. Based on these results, 5 mg/ml was selected as the optimum heparin concentration for the fixation of the positively charged liposomes.

3.2. Stability assay of heparin-liposomes in serum

The change in the size of the liposomes in serum is clearly related to the adsorption of the serum protein on the liposomal surface (Sahli et al., 1998; Lin et al., 1997). The change in the size of the liposomes with time in serum at 37 °C is shown in Fig. 2. The average size of the control liposomes increased greatly from 87 to 270 nm after 20 h of incubation in serum. In

Table 1
Characteristics of the various liposomes

Composition	Size (nm)	Zeta potential (mV)	Loading efficiency (%)
Conventional liposomes (HSPC:CHOL)	87.6 ± 2.4	−3.88 ± 1.4	89
PEG-liposomes (HSPC:CHOL: DSPE-mPEG-2000)	96.3 ± 0.1	−8.63 ± 4.3	91
Cationic liposomes (HSPC:CHOL: DDAB)	91.2 ± 1.5	14.02 ± 2.6	91
Heparin-liposomes (HSPC:CHOL: DDAB:Heparin)	101.2 ± 0.5	−70.12 ± 0.7	91

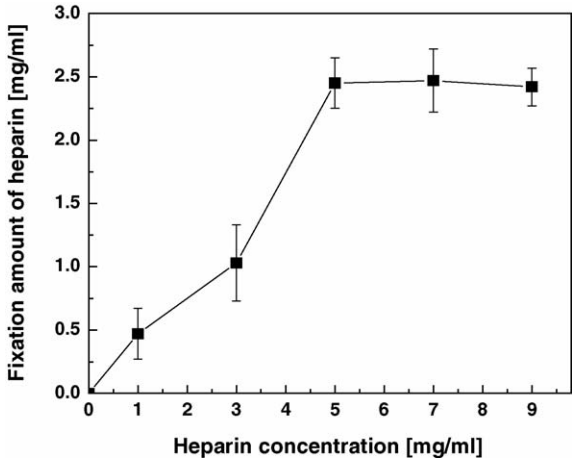

Fig. 1. Amount of heparin conjugated on the surface of the positively charged liposome according to the concentration of heparin solution. The positively charged liposome was composed of HSPC:CHOL:DDAB. The amount of heparin was measured by the Toluidine blue assay method. The data represent the mean ± S.D. ($n = 3$).

the case of the heparin-liposomes, the liposome size scarcely increased after 60 h of incubation. Furthermore, the change in the size of the heparin-liposomes was even smaller than that of the PEG-liposomes ($^{*}P < 0.001$ versus PEG-liposomes, $^{**}P < 0.001$ versus control liposomes). These results indicate that heparin conjugated to the liposomal surface can reduce the interactions of the heparin-liposomes with proteins in serum, due to the high flexibility and hydrophilicity of the heparin molecules.

3.3. Pharmacokinetics in tumor-bearing mice

The profiles of the DOX concentration in the bloodstream after the i.v. injection of the control liposomes, PEG-liposomes and heparin-liposomes at a dose of 6 mg DOX/kg body weight are shown in Fig. 3. The free DOX rapidly disappeared from the circulation within 2 h due to its short half-life ($t_{1/2}$; 1.68 h).

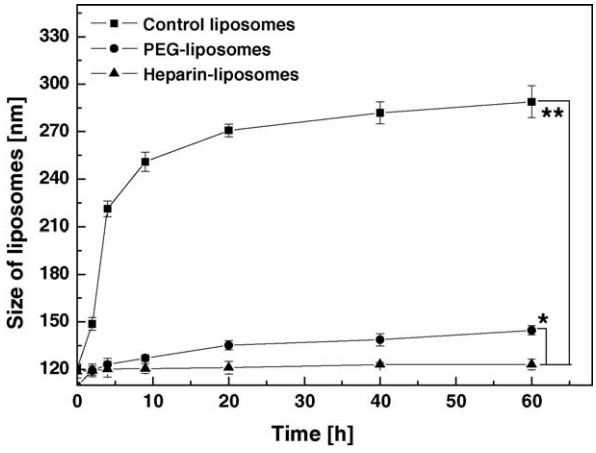

Fig. 2. Change of liposome size during incubation in 50% (v/v) serum at 37 °C ($^{*}P < 0.001$ vs. PEG-liposomes, $^{**}P < 0.001$ vs. control liposomes). The data represent the mean ± S.D. ($n = 3$).

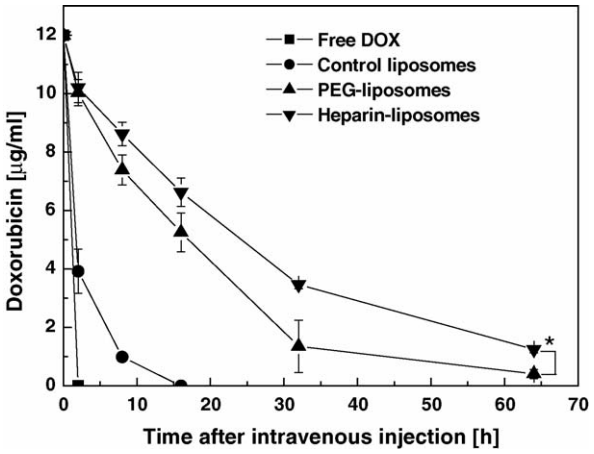

Fig. 3. Pharmacokinetic profiles of DOX encapsulated in various liposomal formulations after intravenous injection in tumor-bearing mice at a single dose of 6 mg/kg ($^{*}P < 0.001$ vs. PEG-liposomes). The data represent the mean ± S.D. ($n = 3$).

In contrast, all of the DOX-loaded liposomes showed a longer circulation time than the free DOX. The DOX concentration of the heparin-liposomes in the bloodstream was much higher ($t_{1/2}$; 19.80 h, $^{*}P < 0.001$ versus PEG-liposomes) than that of the control or PEG-liposomes at each sampling time. These results suggest that heparin-liposomes can prolong the circulation time of DOX. These profiles confirm that heparin is effective at improving the liposomal stability in the bloodstream. Notably, the heparin which was fixed on the liposomal surface sheltered DOX from the serum proteins and prolonged its half-life as compared with those of the free DOX, control ($t_{1/2}$; 2.37 h) or PEG-liposomes ($t_{1/2}$; 12.80 h).

The pharmacokinetic parameters of the various liposomal DOX formulations are summarized in Table 2. The heparin-liposomes significantly increased the half-life ($t_{1/2}$; 19.80 h), mean residence time (MRT; 28.42 h) and area under the curve (AUC; 319.59 μg h/ml) of DOX in circulation, as compared with the PEG-liposomes ($t_{1/2}$; 12.80 h, MRT; 17.78 h, AUC; 201.24 μg h/ml, $^{*}P < 0.01$ versus heparin-liposomes) and free DOX ($t_{1/2}$; 1.68 h, MRT; 1.95 h, AUC; 12.00 μg h/ml $^{**}P < 0.01$ versus heparin-liposomes). These results indicate that heparin-liposomes can circulate for a longer time in the blood circulation system than free DOX and PEG-liposomes.

Table 2
Pharmacokinetic parameters of DOX after i.v. injection of the various liposomal formulations in tumor-bearing mice at a dose of 6 mg DOX/kg ($n = 3$)

	Free DOX*	Control liposomes	PEG-liposomes**	Heparin-liposomes
AUC (μg h/ml)	12.00	25.59	201.24	319.59
$t_{1/2}$ (h)	1.68	2.37	12.80	19.80
CL (ml/h)	17.4	4.62	0.60	0.38
MRT (h)	1.95	4.82	17.78	28.42

AUC, area under the curve; $t_{1/2}$, half-life time; CL, clearance; MRT, mean residence time.

* $P < 0.001$ vs. heparin-liposomes.

** $P < 0.003$ vs. heparin-liposomes.

3.4. Tissue distribution study

The prolonged systemic circulation time of the heparin-liposomes could be attributed to a reduction in the RES uptake of the liposomes in the liver and spleen. To clarify the reduction in the RES uptake of the polymer-fixed liposomes, the DOX concentrations in the tissues were measured after the i.v. injection of free or liposomal DOX solution. The DOX concentrations in the tumor, heart, liver and spleen are shown in Fig. 4. The concentrations of liposomal DOX in the tissues were significantly higher than those of free DOX except in the heart ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). Notably, the DOX concentration of the heparin-liposomes in the tumor was significantly higher than those of the other liposomal groups at 16 h (Fig. 4A) ($^{*}P<0.05$, $^{**}P<0.01$). The DOX concentration of the heparin-liposomes in the heart was much lower at 2 h than those of free DOX and the control liposomes (Fig. 4B). Drug levels in the heart are closely related to the inherent cardiac toxicity of DOX. Therefore, using liposomal DOX formulations could reduce the cardiac toxicity of DOX. The DOX concentrations of the heparin-liposomes in the liver were significantly lower than those of the control liposomes (Fig. 4C). These results clearly showed that the RES uptake of the liposomes could be reduced by the fixation of heparin to the liposomes. However, the concentrations of the liposomal DOX in the spleen were not significantly different (Fig. 4D).

3.5. Antitumor activity

The antitumor activities of the DOX-loaded liposomes were evaluated in B16F10 melanoma tumor-bearing mice (six per group) at a dose of 6 mg DOX/kg body weight. As shown in Fig. 5, all of the liposomal DOX formulations suppressed the growth of the tumor as compared with the PBS control group. In particular, the heparin-liposomes showed higher inhibition of tumor growth ($^{*}P<0.003$ versus PBS, $^{**}P<0.013$ versus control liposomes, $^{***}P<0.006$ versus PEG-liposomes) than the other groups. This higher tumor therapeutic efficacy of the heparin-liposomes was consistent with the higher tumor accumulation of the heparin-liposomes as compared with the other groups (Fig. 4A). It is reasonable to suppose that the

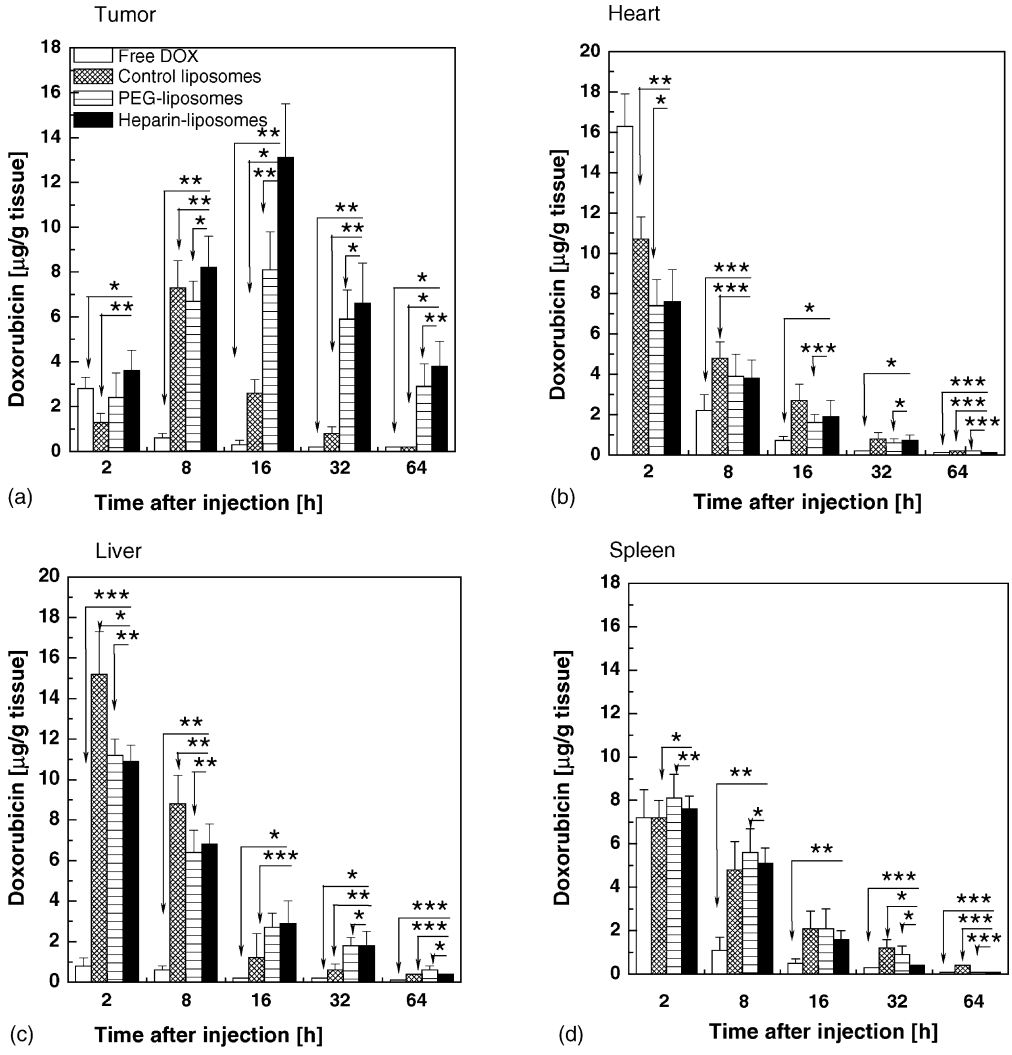

Fig. 4. Tissue distribution of DOX after intravenous injection of DOX-loaded liposomal formulations in C57BL/6 mice at a single dose of 6 mg/kg ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). Each point represents the mean of three mice ±S.D. ($n=3$).

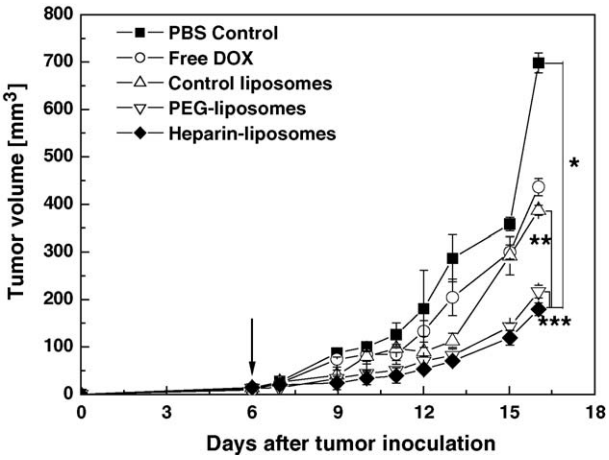

Fig. 5. Tumor growth inhibition by injection of various liposomal DOX formulations in tumor-bearing C57BL/6 mice. A 5×10^5 murine B16F10 melanoma cells were inoculated into the armpits of the mice and the animals were treated with an intravenous injection of free DOX, control liposomes, PEG-liposomes, heparin-liposomes or normal PBS solution as a control treatment. The arrow indicates the number of days after the implantation of the B16F10 tumor cells that the various liposomal DOX formulations were injected. ($^{*}P < 0.003$ vs. PBS control, $^{**}P < 0.013$ vs. control liposomes, $^{***}P < 0.006$ vs. PEG-liposomes). The data represent the mean of six mice ±S.D.

higher DOX levels of the heparin-liposomes in the tumor would result in higher therapeutic potency. Although some side effects of polymeric modifiers might be expected, no severe toxicities, such as fever, loss of weight, cachexia and myalgia, were observed in the treated mice.

4. Discussion

The use of heparin-incorporated liposomes as carriers for anticancer drugs has not been developed or evaluated in preclinical models. To enhance the circulation time of DOX-loaded liposomes in the bloodstream and hence their antitumor activity, we studied the surface modification of the liposomal membrane with heparin. Most of the previous studies of DOX focused on prolonging the circulation time of the liposomes in order to reduce its side effects, such as its myelosuppression and dose-limiting cardiotoxicity (Gabizon et al., 1993; Xiong et al., 2005b). The use of polymer-fixed liposomes was designed to increase the circulation time of DOX as well as to prevent RES uptake in the spleen and liver (Takeuchi et al., 2000). In our study, heparin, which has anticoagulant properties in the blood, was applied to the liposomes in order to enhance their stability and, consequently, to prolong their circulation time after i.v. injection. Heparin was simply conjugated on the surface of the liposomes via a strong ionic interaction (Table 1). As anticipated, the stability of the heparin-liposomes in vitro was increased due to inhibition of protein adsorption to the liposomal surface. This enhanced stability was confirmed by the maintenance of the size of the liposomes in serum (Fig. 2).

The clearance and tissue distribution of sterically stabilized liposomes have been studied by a number of research groups (Charrois and Allen, 2004; Junping et al., 2000). In general, polymer fixation on the liposomal surface effectively protects liposomes from interacting with plasma proteins in the blood, leading to both a reduction of the RES uptake and prolongation of the circulation time. In our study, the prolonged circulation time of the heparin-liposomes was observed as compared with the other liposomal groups (Fig. 3). These results indicate that the circulation time of the liposomes can be prolonged by the fixating heparin to them, such as by modifying the liposomes with PEG. Moreover, the present results also confirm that heparin fixation is effective at improving the stability of the liposomes in the bloodstream. The liposomal DOX formulation used in the pharmacokinetics study significantly increased its AUC, half-life and MRT, as compared with the free DOX solution. The CL of the liposomal DOX was significantly decreased, as compared with that of free DOX (Table 2). In particular, the heparin-liposomes showed the highest value of the AUC, half-life and MRT.

In the analysis of the data concerning the DOX concentration in the liver after the i.v. injection, a statistically significant difference was observed at all sampling time intervals (Fig. 4C). The lower DOX concentration in the liver in the case of the heparin-liposomes as compared with the control liposomes indicates a reduction in the RES uptake due to the fixation of the liposomes with heparin. This reduction in the RES uptake of the sterically stabilized liposomes can be attributed to the reduced binding of plasma proteins to the liposomes. The heparin-liposomes would be expected to be sufficiently sterically stable to protect the liposomes from the unfavorable adsorption of proteins under biological conditions. Considering the sterical stabilization effect of the fixed polymer, both the hydrophilicity and flexibility of the polymer would likely be responsible for reducing the RES uptake of the liposomes. These properties were reflected in the longer retention of the polymer-fixed liposomes in the bloodstream. Generally, DOX tends to be taken up by the heart, resulting in severe cardiac toxicity. Therefore, the use of drug carriers including liposomes is expected to reduce this cardiac toxicity by controlling the distribution of DOX. From the analysis of the DOX concentrations in the heart after the i.v. injection, the encapsulation of DOX into the liposomes was found to significantly reduce the uptake of DOX by the heart (Fig. 4B). Notably, the DOX concentration in the heart in the case of the control liposomes was higher than that observed with the PEG- or heparin-liposomes. This result is presumably related to the low stability of the control liposomes, resulting in the adsorption of the serum proteins not being inhibited and, consequently, the blood circulation time of the liposomes not being significantly prolonged. Therefore, DOX would tend to be released from the control liposomes during the circulating period and this might explain its greater accumulation in the heart than was observed in the other liposomal groups (Lu et al., 2004). Consequently, the uptake of DOX in the heart tissue in the case of the control liposomes was similar to that of free DOX.

The polymer-fixed liposomes with their long-circulating properties were able to extravasate into the extracellular spaces of a solid tumor through the permeable endothelial barriers in the vascularized tumors (Takeuchi et al., 2001; Wu et al., 1993). In the results of a biodistribution study, the heparin-liposomes demonstrated the highest DOX accumulation in the

tumor (Fig. 4A). Thus, the difference in the accumulation of the various liposomal DOX formulations observed in this study would inevitably be related to their intracellular uptake by the tumor cells. The increased accumulation of liposomal DOX in the tumor tissue, including that of the polymer-fixed liposomes, reflects the enhanced permeability and retention (EPR) effects caused by the polymer fixation (Takeuchi et al., 2001; Maeda et al., 1992). Notably, the improved circulation time of the polymer-fixed liposomes would result in their having higher affinity to the tumor cells or extracellular spaces and/or increased stability after passing the endothelial layer (Gabizon and Papahadjopoulos, 1988). In addition, heparin is known to have specific binding properties to peptides in B16F10 melanoma cells, and this would be expected to directly promote cell adhesion (Yoshida et al., 1999). Therefore, in the present study, the accumulation of the heparin-liposomes in the tumor was higher than that of the other liposomal groups. For this reason, the heparin-liposomes would be expected to have the highest therapeutic efficacy due to the EPR effect (Fig. 5). The DOX encapsulated within the heparin-liposomes could be released from the liposomes and then diffused passively into the tumor cells, or the liposomes might be directly internalized by endocytosis through the EPR effect via passive targeting (Xiong et al., 2005b; Maeda et al., 1992; Gabizon and Papahadjopoulos, 1988). The higher therapeutic efficacy of the heparin-liposomes can be attributed to the EPR effect via passive targeting, due to the enhancement of the stability and prolonged circulation time of the liposomes caused be the heparin fixation.

In conclusion, sterically stabilized liposomes preventing protein adsorption were developed by making use of the ionic interaction between the negatively charged heparin and the positively charged liposomes. The use of the DOX-loaded heparin-liposomes led to a significant increase in the in vitro stability by inhibiting the adsorption of serum protein to the liposomal surface. Notably, heparin conjugation on the liposomal surface was effective at increasing the tumor accumulation of DOX through the prolongation of the circulation time of the liposomes in B16F10 tumor-bearing mice. In addition, the heparin-liposomes reduced the uptake of DOX in the heart, thereby reducing the severe cardiac toxicity of DOX. These results demonstrated the promising potential of the heparin-stabilized liposomes to improve the therapeutic efficacy of DOX and reduce the DOX-associated systemic toxicity.

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