Enhanced Delivery and Antitumor Activity of Doxorubicin Using Long-Circulating Thermosensitive Liposomes Containing Amphipathic Polyethylene Glycol in Combination with Local Hyperthermia

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Enhanced delivery of doxorubicin (DXR) to a solid tumor subjected to local hyperthermia was achieved by using long-circulating, thermosensitive liposomes (TSL) composed of dipalmitoyl phosphatidylcholine (DPPC)/distearoyl phosphatidylcholine (DSPC) (9:1, m/m) and 3 mol% amphipathic polyethylene glycol (PEG) in colon 26-bearing mice. Inclusion of 3 mol% of distearoyl phosphatidylethanolamine derivatives of PEG (DSPE-PEG, amphipathic PEG) with a mean molecular weight of 1000 or 5000 in DPPC/DSPC liposomes resulted in decreased reticuloendothelial system (RES) uptake and a concomitant prolongation of circulation time, affording sustained increased blood levels of the liposomes. Concomitantly, DXR levels in blood were also kept high over a long period. The presence of amphipathic PEG did not interfere with the encapsulation of DXR by the pH gradient method (>90% trapping efficiency) or with the temperature-dependent drug release from the liposomes. The optimal size of these liposomes was 180–200 nm in mean diameter for thermosensitive drug release and prolonged circulation time. The DXR levels in the tumor after injection of long-circulating TSL (DXR-PEG1000TSL or DXR-PEG5000TSL, at a dose of 5 mg DXR/kg) with local hyperthermia were much higher than after treatment with DXR-TSL lacking PEG or with free DXR, reaching 7.0–8.5 DXR μg/g tumor (approximately 2 times or 6 times higher than that of DXR-TSL or free DXR, respectively). Furthermore, the combination of DXR-PEGTSL and hyperthermia effectively retarded tumor growth and increased survival time. Our results indicate that the combination of drug-loaded, long-circulating, thermosensitive liposomes with local hyperthermia at the tumor site could be clinically useful for delivering a wide range of chemotherapeutic agents in the treatment of solid tumors.

KEY WORDS: liposome; thermosensitive liposome; long-circulating liposome; drug delivery system; hyperthermia; doxorubicin.

INTRODUCTION

The therapeutic efficacy of antitumor drugs is restricted by dose-limiting toxicities to normal tissues *in vivo*, but would be greatly improved if the drugs could be selectively directed to the tumor sites and away from sensitive tissues such as heart and bone marrow (1). Liposomes have been proposed to be useful drug carriers for targeted drug delivery systems, and are under investigation in several therapeutic fields (2). In order to achieve maximum targeting, liposomes should remain in the systemic circulation for a long time (2). However, formulations of liposomes used in the past were rapidly removed from the circulation by the reticuloendothelial system (RES) (3,4). Newly developed liposomes, containing either monosialoganglioside GM1 (GM1) (5) or amphipathic polyethylene glycol (PEG) (6–8), can evade RES uptake, thereby achieving prolonged half-lives in the circulation, and have been used to deliver cytotoxic drugs selectively to tumors (2,9).

Liposomes can be designed to release their contents preferentially in an anatomical region subjected to local hyperthermia (thermosensitive liposomes) (10,11). We have recently reported the formation of long-circulating thermosensitive liposomes entrapping DXR, which contained GM1 as functional molecules for prolonging their circulation time, and we demonstrated enhanced delivery and antitumor activity of DXR to colon 26 solid tumor of mice by using these liposomes in combination with local hyperthermia (12). However, it is difficult to obtain large quantities of GM1 either by extraction from natural sources or by synthesis, so GM1-containing liposomes are impractical for therapeutic applications. On the contrary, amphipathic PEG is particularly useful because of its ease for preparation, relatively low cost, controllability of the molecular weight and linkability to lipid by a variety of methods.

We now describe the application of amphipathic PEG for the formulation of thermosensitive liposomes with prolonged circulation time and controlled release of an entrapped drug at a hyperthermic site *in vivo*. To establish the optimum conditions, the efficiency of DXR encapsulation and the thermosensitivity of liposomes containing PEG of different molecular weights were examined from the viewpoint of effective drug release in response to hyperthermia, and the drug biodistribution and chemotherapeutic activity of entrapped DXR were evaluated in colon 26 tumor-bearing mice subjected to local hyperthermia at the tumor site.

MATERIALS AND METHODS

Materials

Dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC) and distearoylphosphatidylethanolamine (DSPE) were kindly donated by NOF Corp., Tokyo. A series of monomethoxy poly(ethylene glycol) succinimidyl succinate (PEG-OSu), with average molecular weights of 1000–12000, were also provided by NOF Corp. [^{3}H]Cholesteryl hexadecyl ether, ^{67}Ga and ^{51}Cr were from New England Nuclear. Doxorubicin (DXR) was a kind gift from Kyowa Hakko, Tokyo. Deferoxamine was purchased from Ciba Geigy, Kobe. A series of amphipathic PEGs were synthesized systematically by combination of

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DSPE with PEG-OSu, as described previously by Maruyama et al. (13).

Liposome Preparation and DXR Encapsulation

Liposome preparation (DPPC/DSPC (9:1, m/m) and an appropriate amount of DSPE-PEG derivative with various molecular weight) and DXR encapsulation were described previously (12). The lipid mixture (5 mg in total lipids) was dissolved in 600 μl of isopropyl ether/chloroform (1:1, v/v) and 300 μl of 300 mM citric acid (pH 4.0) were added. LUV was formed by the reverse-phase evaporation method and extruded through Nuclepore polycarbonate filters to control size. Liposome size was measured by a Nicomp 370 submicron particle analyzer (HIAC Pacific Scientific). An appropriate amount of ^{67}Ga-DF (14) or [^{3}H]cholesteryl hexadecyl ether was added for estimation of biodistribution or integrity of liposome *in vivo*, respectively. The encapsulation of DXR into liposomes was done under fixed conditions (15); i.e., a difference pH of 3.8 between the inside and outside of the liposome membrane, a DXR/lipid weight ratio of 0.2, and a standing time of 10 min at 60°C at the loading step. The amount of liposomally entrapped DXR was determined with a fluorescence spectrometer (Hitachi F-3000) by diluting liposomes with 0.3 N HCl-50% ethanol and measuring the fluorescence intensity (Ex: 470 nm, Em: 590 nm).

Thermosensitivity of Liposomes

Temperature-dependent release profiles of DXR from TSL were estimated by incubation of the liposomes at various temperatures as follows. The DXR-liposomes were diluted with saline or 20% (v/v) fetal bovine serum. One ml of each sample was placed in a water bath (HAAKE F2) and heated at 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C or 43°C. The released DXR was separated from the liposomal suspension by ultrafiltration and assayed by fluorescence spectrometry as described under doxorubicin encapsulation. A Centrisart (molecular weight cut-off 20,000, Sartorius, 3000 rpm, 20 min) was used for this procedure. The absorption of DXR to membrane was less than 2%.

Biodistribution and Stability *in vivo*

Mouse colon carcinoma 26 cells (1×10^5 cells) were inoculated into the hind foot of male Balb/c mice (male, 8 weeks old, and weighing 22–25 g), and the tumor was allowed to grow for approximately 8 days, when the mean of its length and width was 8 mm. DXR-liposomes labeled with ^{67}Ga-DF or [^{3}H]cholesteryl hexadecyl ether were injected via the tail vein into the colon 26 solid tumor-bearing mice at a dose of 5 mg DXR/kg. At different times after injection, mice were anesthetized, bled via the retro-orbital sinus and killed by cervical dislocation. Organs were excised and counted for ^{67}Ga using a gamma counter. Scintillation counting was done by the procedure described previously (12). The weight of total blood was assumed to be 7.3% of the body weight (16). Blood concentration as back ground in each organ was corrected by examining the distribution of ^{51}Cr-labeled erythrocytes (12).

In vivo Studies with Hyperthermia

The colon 26 solid tumor-bearing mice were anesthetized with nembutal. The free DXR or liposomal DXR was injected intravenously via the tail vein at a dose of 5 mg DXR/kg. Local hyperthermia using a radiofrequency oscillator (RF-hyperthermia HEH-100, Omron, Kyoto) with a 42°C heating temperature was started at 5 min after drug administration and was continued for 20 min. The hyperthermia apparatus used in this study was modified to accommodate small animals. At 5 min after completion of hyperthermia treatment, blood was collected from the retro orbital sinus and major organs were excised. A 0.1 g portion of tissue, or the whole tissue if it weighed less than 0.1 g, was used for measurement of DXR concentration. Samples were homogenized and extracted with butanol/toluene (1:1, v/v), then the extracts were subjected to HPLC assay according to the previous method (12).

Tumoricidal Effect

Tumor-bearing mice were assigned at random into groups of 10. Treatment was started when the tumor had reached a diameter of 8 mm (= Day 0). The dose per injection was 5 mg DXR/kg body weight. Local hyperthermia at 42°C for 20 min was done at 5 min after injection under light anesthesia. Tumor volumes were determined by a reported method (17). Survival time was recorded in days after treatment with local hyperthermia.

RESULTS

Effect of PEG on the Encapsulation of DXR into DPPC/DSPC Liposomes by pH Gradient Method

We first examined the effect of concentration and molecular weight of amphipathic PEG on the encapsulation of DXR in DPPC/DSPC liposomes (Table I). The mean diameters of all liposome preparations were controlled to within the range of 180–200 nm. DXR was entrapped into liposomes with >90% efficiency, even in the presence of 3 mol% DSPE-PEG derivatives, except in the case of PEG with a mean molecular weight of 12000. Though the ordinary method described in our previous report gave only 20% trapping efficiency (18, data not shown), the pH gradient method employed in this study was more effective for the encapsu-

Table I. Effect of PEG on pH-Dependent DXR Uptake into Thermosensitive Liposomes (DPPC/DSPC)[a]

Liposomal composition	Molar ratio	Diameter (nm (SD))	Trapping efficiency (%)
(DPPC/DSPC)	9:1	182.9 (44)	>98
PEG1000/DPPC/DSPC	0.31:9:1	194.1 (64)	>95
PEG1000/DPPC/DSPC	0.61:9:1	195.6 (76)	60–65
PEG2000/DPPC/DSPC	0.31:9:1	190.6 (60)	95
PEG2000/DPPC/DSPC	0.61:9:1	193.5 (66)	63
PEG5000/DPPC/DSPC	0.31:9:1	195.0 (72)	>90
PEG5000/DPPC/DSPC	0.61:9:1	195.8 (76)	25–28
PEG12000/DPPC/DSPC	0.31:9:1	198.7 (87)	32

[a] 2.0 mg of DXR was added to liposome solution (10 mg total lipids).

lation of DXR into TSL. However, the inclusion of 6 mol% DSPE-PEG derivatives or 3 mol% PEG12000 resulted in decreased trapping efficiency. These results clearly indicated that the transmembrane incorporation of DXR into TSL by the pH gradient method is influenced by the amount and the molecular weight of amphipathic PEG.

Liposomes lacking PEG precipitated during storage in a refrigerator for 1 day, owing to aggregation. On the other hand, no aggregation was observed, even after 5 days, in liposomes prepared using PEG derivatives. The improved dispersion of these liposomes was considered to be due to the increased hydrophilicity of the liposome surface.

On the basis of these results, 3 mol% of PEG1000, PEG2000 or PEG5000 was considered optimal. Three types of DXR-encapsulating liposomal formulations, i.e., PEG1000/DPPC/DSPC, PEG5000/DPPC/DSPC and DPPC/DSPC, were therefore employed in subsequent studies.

Thermosensitivity

Temperature-dependent release of DXR from TSL (180–200 nm in mean size) containing 3 mol% of amphipathic PEG was examined in saline. As shown in Fig. 1, 43.2% of the entrapped DXR was released from liposomes lacking PEG by incubation at 42°C for 5 min with saline. On the other hand, inclusion of PEG1000 and PEG5000 in TSL gave a higher DXR release at 42°C: DXR release levels were 57.5% and 78.2%, respectively. PEG5000TSL was leaky at temperatures below 40°C as compared with PEG1000TSL. It was observed that the DXR release in 20% fetal bovine serum was almost the same as that in saline (data not shown). Interestingly, smaller sized vesicles (about 110 nm in mean diameter) in DPPC/DSPC liposomes and PEG1000/DPPC/DSPC liposomes showed no thermosensitivity at all (Fig. 1). These results indicate that DXR release from TSL containing amphipathic PEG is markedly influenced by the molecular weight of amphipathic PEG and the liposomal size.

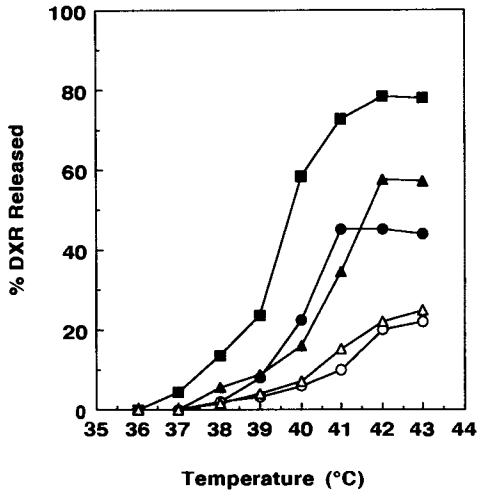

Fig. 1. Effect of inclusion of amphipathic PEG and liposomal size on temperature-dependent release of DXR from TSL (DPPC/DSPC, 9:1 m/m, (●) 189 nm, (○) 118 nm) or PEG-TSL (PEG/DPPC/DSPC, 0.31:9:1 m/m, (▲) PEG1000 and 191 nm, (△) PEG1000 and 121 nm, (■) PEG5000 and 188 nm). DXR liposomes (10 μg DXR/ml) were incubated in saline for 5 min.

The effect of PEG derivatives on the fluidity of DPPC/DSPC liposomes was determined by measuring the fluorescence anisotropy of diphenylhexatriene (DPH) in liposomes according to a reported method (19). The inclusion of 3 mol% of PEG5000 decreased the anisotropy of DPH in DPPC/DSPC liposomes, while PEG1000 had no effect. Thus, PEG5000 increased the membrane fluidity of DPPC/DSPC liposomes.

Biodistribution of DXR-PEG/DPPC/DSPC Liposomes

DXR-TSL, DXR-PEG1000TSL and DXR-PEG5000TSL (180–200 nm in mean size) were labeled with ^{67}Ga-DF and injected i.v. into mice via the tail vein. The biodistribution was measured up to 3 h after injection. As can be seen in Fig. 2, TSL lacking PEG were rapidly cleared from the blood circulation and concomitantly accumulated in the RES (liver), suggesting that liposomes of this lipid composition and size range are readily taken up by the RES. On the other hand, the inclusion of 3 mol% of DSPE-PEG derivatives significantly increased the blood levels of TSL over a long time and decreased RES uptake. These effects were almost the same as those previously reported for TSL containing 6 mol% of GM1 (12). However, the circulation time of PEG/DPPC/DSPC thermosensitive liposomes was less prolonged than that of egg PC/CH or DSPC/CH liposomes containing 3 mol% of DSPE-PEG derivatives, described previously (7,13).

The stability of DXR-PEG1000TSL *in vivo* was estimated as a DXR-to-lipid ratio (wt/wt) (Fig. 2). The drug-to-lipid ratio of DXR-PEG1000TSL prior to injection was 0.19. The drug-to-lipid ratio of plasma samples obtained by 1 h after injection was 0.19, falling to 0.12 at 3 h. Thus, DXR was released from PEG1000TSL spontaneously from 1 h after injection.

We also followed the DXR release *in vivo* under conditions of hyperthermia for 20 min, from 5 min after liposome injection. A drug-to-lipid ratio of 0.13 was obtained soon after hyperthermia, and the value dropped to 0.06 at 1 h after injection (at 35 min after the end of hyperthermia). These data suggest that PEG1000TSL effectively release entrapped DXR in a thermosensitive manner *in vivo* in the early phase, but subsequently become leaky.

DXR Distribution in Mice

The blood and tissue-associated DXR levels were measured after injection of DXR-loaded thermosensitive liposomes, with or without local hyperthermia. As shown in Fig. 3(A), DXR levels in blood after administration of DXR-PEG1000TSL and DXR-PEG5000TSL were significantly increased as compared with the other formulations. These results were due to the high blood level and low RES uptake of TSL containing PEG. After administration as a solution, DXR disappeared from blood very rapidly. DXR-TSL was also cleared rapidly from blood due to high RES uptake (Fig. 2). When hyperthermia was applied (Fig. 3(B)), the blood levels of DXR were decreased, indicating that DXR was released from TSL containing PEG *in vivo* in a thermosensitive manner and released DXR was cleared rapidly from the blood.

There were no marked differences in tissue levels of

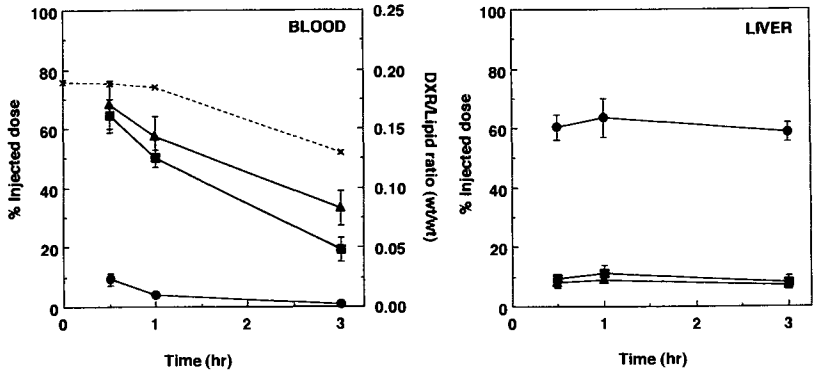

Fig. 2. Time course of blood residence and RES (liver) uptake of DXR-TSL (●), DXR-PEG1000TSL (▲) and DXR-PEG5000TSL (■) in colon 26 solid tumor-bearing mice without hyperthermia. Tumor-bearing mice were i.v. injected with 0.2 ml of ^{67}Ga-labeled liposomes (35 kBq and 600 μg lipids). The sumbol X indicates the DXR-to-lipid ratio after administration of DXR-PEG1000TSL. Liposomes labeled with [^{3}H] cholesteryl hexadecyl ether (20 kBq) were injected via the tail vein at a dose of 5 mg DXR/kg.

DXR between groups with and without hyperthermia. For DXR-TSL formulations lacking PEG, high levels of DXR were observed in liver (52.5 μg/g) and spleen (98.5 μg/g), presumably as a result of accumulation of DPPC/DSPC liposomes therein (Fig. 2). On the other hand, the distribution of DXR to liver and spleen after injection of DXR-PEG1000TSL or DXR-PEG5000TSL was significantly decreased. Thus, the blood level and the tissue distribution characteristics of DXR reflected those of the liposomes themselves. There was no marked difference in the accumulation of DXR in the heart among free drug and liposomal formulations.

The tumor-associated DXR levels with or without hyperthermia are summarized in Fig. 4. Without hyperthermia, there was no marked difference in tumor accumulation of DXR at 30 min after injection among liposomal DXR formulations. However, liposomal DXR formulations tended to show higher accumulation than DXR solution. On the other hand, the system of DXR-PEG1000TSL or DXR-PEG5000TSL, and hyperthermia gave a value of 1.8-fold or 1.9-fold higher than that of DXR-TSL and hyperthermia, and 5.3-fold or 5.6-fold higher than that of free drug solution and hyperthermia at 30 min after injection, respectively. These levels with the liposomal systems were, of course, much higher than those obtained without hyperthermia. Since amphipathic PEG prolonged the circulation time of liposomes, thereby increasing the amount of liposomes which pass the heating site, the amount of DXR released from PEGTSL at the heating site is expected to be higher than that from TSL lacking PEG. Thus, we consider that the entrapped DXR was efficiently released from PEG-thermosensitive liposomes by hyperthermia at the tumor site and entered the tumor tissue by simple diffusion.

Antitumor Activity

The antitumor activities of DXR solution and ther-

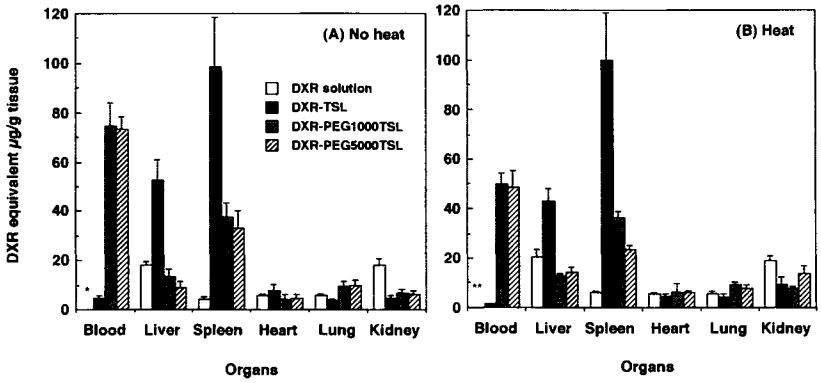

Fig. 3. Tissue DXR levels after the administration of free or thermosensitive liposomal DXR to colon 26 solid tumor-bearing mice with (panel B) or without (panel A) local hyperthermia. Symbols are defined in panel A. DXR solution or liposomal DXR was injected intravenously via the tail vein at a dose of 5 mg/kg. Local hyperthermia with 42°C heating temperature was applied at the tumor site 5 min after drug administration and was continued for 20 min. DXR concentrations in major organs were assayed at 5 min after termination of hyperthermia. * and ** are 0.072 and 0.071 μg/ml, respectively.

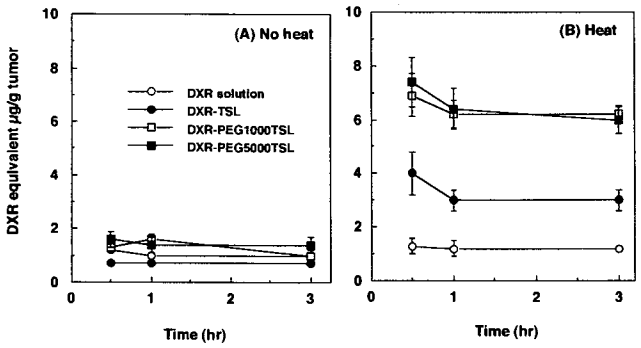

Fig. 4. Tumor DXR levels after the administration of free DXR or thermosensitive liposomal DXR to colon 26 solid tumor-bearing mice with (panel B) or without (panel A) local hyperthermia at the tumor site. Symbols are defined in panel A.

mosensitive liposomal DXR with hyperthermia are summarized in Table II. Hyperthermia alone (without administration of drugs) caused tumor growth retardation but gave no improvement in survival time, compared with the control. The system of free drug and hyperthermia was ineffective, giving the same result as heating alone. On the other hand, the combinations of systemic liposomal DXR injection and local hyperthermia effectively retarded tumor growth and increased survival time. These results show clearly that DXR encapsulated in long-circulating, thermosensitive liposomes, in combination with hyperthermia, causes a marked improvement in therapeutic efficacy, inhibiting the tumor growth.

DISCUSSION

Weinstein et al. (10,11) reported that temperature-sensitive liposomes injected i.v. leak their contents preferentially in an anatomic region subjected to local hyperthermia. Although thermosensitive liposomes could be useful for treatment of selected tumors in combination with local hyperthermia, they have drawbacks such as low stability and short half-life in the blood. We found that GM1-containing thermosensitive liposomes exhibited a prolonged circulation time, and could be utilized effectively for thermosensitive, selective liposomal drug delivery to tumors in combination with hyperthermia (12). We were interested to see whether amphipathic PEG affords similar effects, since PEG has significant advantages over GM1 in terms of cost and ease of preparation.

The present results clearly indicated that the use of PEG-TSL containing DXR in combination with local hyperthermia could deliver a higher drug concentration to the solid tumor (Fig. 4) and resulted in superior therapeutic efficacy as compared with the PEG-lacking thermosensitive liposomal system used in the past (Table II). Although there were only small differences in the release rate of DXR at 42°C between TSL lacking PEG and long-circulating TSL (Fig. 1), the drug concentration in the tumor provided by long-circulating TSL was about 2 times higher than that by TSL lacking PEG. We presume that since a much larger amount of long-circulating TSL would pass the heating site during hyperthermia for 20 min compared with TSL lacking PEG, a larger amount of DXR was released at the tumor site by the former liposomes, and diffused into the tumor tissues. Then, the drug was able to diffuse into the tumor cells.

It is known that the long-circulating activity of amphipathic PEG-containing liposomes was dependent on the liposomal size. We and others have shown that PEG-liposomes larger than 300 nm in diameter did not have long-circulating activity (15,20). On the other hand, large liposomes were required for thermosensitive drug release between 40°C and 42°C. For example, large unilamellar vesicles (LUV) of 850 nm could release 85% of entrapped DXR at 41°C within 5 min (21). The LUV showed sharp increase in Ara-C or cisplatin release near the phase transition temperature, while small liposomes exhibited the low release rates (22,23). The lamellar structure of liposome membrane composed of DPPC/DSPC (9:1, m/m) at the gel-to-liquid crystalline transition phase was somewhat loose and porous, but its permeability for DXR was dependent on the size of

Table II. Effects of Thermosensitive Liposome-Encapsulated DXR with Hyperthermia (42°C, 20 min) on Tumor Growth and Survival of Mice Bearing Colon Carcinoma 26 Solid Tumor

Treatments	Tumor volume (cm^3)[a]		Survival time[a]	
	Day 7	Day 14	Mean (day)	%ILS[b]
Control	3.0 (0.7)	6.9 (1.5)	29.4 (4.2)	—
Heat	1.7 (0.4)	4.8 (0.7)**	30.1 (4.5)	2.4
DXR solution + Heat	1.6 (0.4)	3.0 (1.0)	31.5 (5.6)	7.1
DXR-TSL + Heat	0.9 (0.2)	1.3 (0.8)*	39.8 (8.4)	35.4
DXR-PEG1000TSL + Heat	0.6 (0.1)	0.7 (0.1)*,***	46.0[c]	56.5
DXR-PEG5000TSL + Heat	0.7 (0.2)	0.7 (0.2)*,***	44.3[d]	50.7

Data are mean values (S.D.) of 10 mice per group. *Significantly different from DXR solution + Heat, $P < 0.01$; **No significantly different from DXR solution + Heat; ***Significantly different from DXR-TSL + Heat, $P < 0.05$.

[a] Tumor volume and survival time calculated from post-treatment (day 0).

[b] Increased life span (ILS) was calculated as %ILS = ((Mean survival of treated group)/(Mean survival of control group) − 1) × 100.

[c] Three mice survived over 70 days.

[d] One mouse survived over 70 days.

liposome (Fig. 1). Low rate of the drug release from small liposomes is probably due to the larger lipid-membrane curvature. Thus, for our purpose it is necessary to compromise by sizing PEG-TSL at about 200 nm. Since the capillary permeability of the endothelial barrier in nearly vascularized tumors is significantly greater than that of normal organs, it is possible that small liposomes (less than 100 nm mean diameter) with prolonged circulation half-lives could predominantly pass through the leaky tumor endothelium by passive convective transport (14,24). However, such extravasation occurs less readily with larger liposomes (around 200 nm) of the same size as TSL used in this experiment. Therefore, the present results indicate that a larger amount of DXR was released in capillary of the tumor mass from PEG-TSL by hyperthermia and entered the tumor tissue by simple diffusion.

The mechanism by which PEG prolongs the circulation time of liposomes has not been fully clarified. However, increased hydrophilicity (25) and/or steric barrier on the liposomal surface (6) might be important for long circulating activity. A hydrophilic surface may prevent or reduce the interactions of liposomes with serum constituents, resulting in an enhanced stability of liposomes and a reduced rate of RES interaction. Liposomes with thermosensitivity and long circulating half-lives have a greater chance to release entrapped drug at a hyperthermic site. This system is expected to be clinically useful for delivering a wide range of chemotherapeutic agents in the treatment of solid tumors.

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